Scientific Excellence • Resource Protection & Conservation • Benefits for Canadians
Excellence scientifique • Protection et conservation des ressources • Bénéfices aux Canadiens

The Summer Mortality Syndrome and Haemocytic Neoplasia in Blue Mussels *(Mytilus edulis)* From British Columbia

S. M. Bower

Department of Fisheries and Oceans
Biological Sciences Branch
Pacific Biological Station
Nanaimo, British Columbia V9R 5K6

July 1989

Canadian Technical Report of Fisheries and Aquatic Sciences No. 1703

Fisheries and Oceans Pêches et Océans

Canada

Canadian Technical Report of Fisheries and Aquatic Sciences

Technical reports contain scientific and technical information that contributes to existing knowledge but which is not normally appropriate for primary literature. Technical reports are directed primarily toward a worldwide audience and have an international distribution. No restriction is placed on subject matter and the series reflects the broad interests and policies of the Department of Fisheries and Oceans, namely, fisheries and aquatic sciences.

Technical reports may be cited as full publications. The correct citation appears above the abstract of each report. Each report is abstracted in *Aquatic Sciences and Fisheries Abstracts* and indexed in the Department's annual index to scientific and technical publications.

Numbers 1–456 in this series were issued as Technical Reports of the Fisheries Research Board of Canada. Numbers 457–714 were issued as Department of the Environment, Fisheries and Marine Service, Research and Development Directorate Technical Reports. Numbers 715–924 were issued as Department of Fisheries and the Environment, Fisheries and Marine Service, Technical Reports. The current series name was established with report number 925.

Technical reports are available individually by subscription or exchange. Requests for individual reports should include the issuing establishment's code and the report number and title, and must be accompanied by a cheque or money order. Requests for subscription should be addressed to the Publications Unit.

Rapport technique canadien des sciences halieutiques et aquatiques

Les rapports techniques contiennent des renseignements scientifiques et techniques qui constituent une contribution aux connaissances actuelles, mais qui ne sont normalement pas appropriés pour la publication dans un journal scientifique. Les rapports techniques sont destinés essentiellement à un public international et leur diffusion est mondiale. Ils traitent de tous les sujets scientifiques relevant du mandat du ministère des Pêches et des Océans, c'est-à-dire des sciences halieutiques et aquatiques.

Les rapports techniques peuvent être cités comme des publications complètes. Le titre exact paraît au-dessus du résumé de chaque rapport. Les rapports techniques sont résumés dans la revue *Résumés des sciences aquatiques et halieutiques*, et ils sont répertoriés dans l'index annuel des publications scientifiques et techniques du Ministère.

Les numéros 1 à 456 de cette série ont été publiés à titre de rapports techniques de l'Office des recherches sur les pêcheries du Canada. Les numéros 457 à 714 sont parus à titre de rapports techniques de la Direction générale de la recherche et du développement, Service des pêches et de la mer, ministère de l'Environnement. Les numéros 715 à 924 ont été publiés à titre de rapports techniques du Service des pêches et de la mer, ministère des Pêches et de l'Environnement. Le nom actuel de la série a été établi lors de la parution du numéro 925.

Les rapports techniques sont publiés à l'échelon régional, mais ils sont offerts à l'échelon national. Les demandes de rapports seront satisfaites par l'établissement auteur dont le nom figure sur la couverture et la page du titre. Les rapports épuisés seront fournis contre rétribution par des agents commerciaux.

Canadian Technical Report of
Fisheries and Aquatic Sciences No. 1703

July 1989

THE SUMMER MORTALITY SYNDROME AND HAEMOCYTIC NEOPLASIA IN BLUE MUSSELS (*Mytilus edulis*) FROM BRITISH COLUMBIA

by

S. M. Bower

Department of Fisheries and Oceans
Biological Science Branch
Pacific Biological Station
Nanaimo, British Columbia V9R 5K6

90-01930/

(c)Minister of Supply and Services Canada 1989

Cat. No. Fs 97-6/1703E ISSN 0706-6457

Correct citation for this publication:

Bower, S. M. 1989. The summer mortality syndrome and haemocytic neoplasia in blue mussels (Mytilus edulis) from British Columbia. Can. Tech. Rep. Fish. Aquat. Sci. 1703: 65 p.

ABSTRACT

Bower, S. M. 1989. The summer mortality syndrome and haemocytic neoplasia in blue mussels (Mytilus edulis) from British Columbia. Can. Tech. Rep. Fish. Aquat. Sci. 1703: 65 p.

In an attempt to identify the cause of the high summer mortalities in blue mussels (Mytilus edulis) over 4 cm in shell length, about 2,000 mussels from 6 locations in British Columbia were examined for haemocytic neoplasia, as well as other infectious diseases. This study consisted of 7 separate investigations comprising 3 field and 4 laboratory studies. The field studies were designed to: 1) monitor mortality and haemocytic neoplasia in mussels from Departure Bay that usually experience over 90% mortality each summer, 2) examine the prevalence of haemocytic neoplasia in mussels from 3 localities that had undergone reciprocal transplants 6 months prior to examination, and 3) periodically examine the prevalence of haemocytic neoplasia in mussels held in pearl nets at 5 localities over a 10 month period. The 4 laboratory studies were designed to examine: 1) the effect of different temperatures and salinities on survival and prevalence of haemocytic neoplasia in mussels during the winter months, 2) the effect of temperature on survival and haemocytic neoplasia in small and large mussels during the summer months, 3) the infectivity of haemocytic neoplasia using syringe passage, and 4) the DNA content of haemocytes from mussels with haemocytic neoplasia. The compiled results indicated that haemocytic neoplasia is an infectious and lethal disease for mussels. However, there was no correlation between the prevalence of this condition and the summer mortality syndrome. There was no other pathogen identified that could account for the summer mortality syndrome. It was concluded that blue mussels in British Columbia have a relatively short life cycle and haemocytic neoplasia is one of the manifestations of senescence. Haemocytic neoplasia may also occur in smaller mussels that are not senescent but under stress, due to factors such as low food availability, as observed in the small mussels held at different temperatures in the laboratory for 175 days. Despite the high summer mortalities at some localities, mussel culture has the potential of becoming a viable industry in British Columbia. Profitability for mussel culture will depend on the selection of an appropriate locality for the culture facility, applying innovative culture techniques, and developing new marketing strategies.

RÉSUMÉ

Bower, S. M. 1989. The summer mortality syndrome and haemocytic neoplasia in blue mussels (Mytilus edulis) from British Columbia. Can. Tech. Rep. Fish. Aquat. Sci. 1703: 65 p.

Dans le but de tenter d'identifier la cause de la forte mortalité estivale chez les moules bleues (Mytilus edulis) dont la coquille a plus de 4 cm de longueur, on a examiné environ 2000 moules provenant de 6 endroits en Colombie-Britannique pour détecter la présence de néoplasie hémocytique et d'autres maladies infectieuses. Les recherches ont comporté 7 études distinctes, à savoir trois études sur le terrain et quatre études en laboratoire. Les études sur le terrain visaient à: 1) surveiller la survie et la présence de néoplasie hémocytique chez les moules de la baie Departure, dont la mortalité estivale dépasse habituellement 90%; 2) examiner la prévalence de la néoplasie hémocytique chez des moules de trois localités ayant subi des transplantations réciproques 6 mois auparavant; 3) examiner périodiquement la prévalence de la néoplasie hémocytique chez des moules retenues dans des filets (pearl nets) en 5 endroits différents et surveiller ainsi la croissance et la mortalité au cours d'une période de 10 mois. Les 4 études en laboratoire avaient pour but: 1) d'examiner l'effet de la température et de la salinité sur la survie des moules et sur la prévalence de la néoplasie hémocytique au cours des mois d'hiver; 2) d'étudier l'effet de la température sur la survie des moules et sur la prévalence de la néoplasie hémocytique chez les moules de petite et de grande taille au cours des mois d'été; 3) de déterminer le pouvoir infectieux de la néoplasie hémocytique en effectuant des repiquages à la seringue; et 4) d'examiner l'ADN des hémocytes des moules bleues atteintes de néoplasie hémocytique. La compilation des résultats a révélé que la néoplasie hémocytique est une maladie infectieuse mortelle chez les moules. Cependant, aucune corrélation n'a été établie entre la prévalence de cette maladie et le syndrome de mortalité estivale. On n'a détecté aucune autre maladie pouvant expliquer le syndrome. On a conclu que les moules bleues de la Colombie-Britannique ont un cycle vital relativement court et que la néoplasie hémocytique est un signe de sénescence. La néoplasie hémocytique peut aussi apparaître chez les moules plus petites qui ne sont pas sénescentes, mais qui sont soumises à un stress, par exemple à une carence de ressources alimentaires, comme on a pu l'observer chez des moules de petite taille gardées en laboratoire, à différentes températures, pendant 175 jours. En dépit de la mortalité estivale élevée des moules en certains endroits, la myticulture pourrait devenir une industrie viable en Colombie-Britannique. Sa rentabilité dépendra de la sélection de

sites appropriés pour l'élevage, de l'application de techniques d'élevage innovatrices et de l'élaboration de nouvelles stratégies de commercialisation.

INTRODUCTION

Blue mussels (Mytilus edulis) at several localities in British Columbia and Washington experience high mortalities from unknown causes during the late summer and fall of the second year of growth (Quayle 1978; Heritage 1983; Emmett 1984; Skidmore and Chew 1985; Emmett et al. 1987). The prevalence of mortalities varies with locality but over 90% of the mussels in some populations are lost just as the mussels approach commercial size. The aetiology was not elucidated during previous investigations into this summer mortality syndrome although it was suggested that the high mortalities were related to reproductive stress (Emmett et al. 1987). A blood cell disorder has been observed in mussels from several localities on the northwest coast of North America (Farley 1969; Mix et al. 1979; Mix 1983; Emmett 1984; Cosson-Manneuy et al. 1984; Elston et al. 1988a, b; Elston and Kent 1988), but its relationship to the summer mortality syndrome has not been established.

In keeping with current terminology, the blood cell disorder is called haemocytic neoplasia. The term neoplasia refers to new growth of abnormal tissue. This definition follows that given by the ICES Working Group on the Pathology and Diseases of Marine Organisms and is based strictly on histological criteria, recognizing that all necessary biological criteria (i.e., irreversible uncontrolled growth, metastases, transplantation and host death) may not have been demonstrated (Alderman and Green 1985).

The purpose of this investigation was: 1) to determine the distribution and seasonal prevalence of haemocytic neoplasia in M. edulis from British Columbia; 2) to examine the correlation between the summer mortality syndrome and the prevalence of haemocytic neoplasia in an attempt to determine if the mortalities are attributable to the neoplasia; 3) to attempt to document the infectivity of haemocytic neoplasia in mussels; and 4) to examine mussels for any other previously unrecognized infectious agents that may be causing the summer mortalities.

The ecological terms used in this report coincide with those suggested by Margolis et al. (1982). Specifically, intensity indicates the percentage of neoplastic haemocytes in a sample of haemolymph from an individual mussel, prevalence denotes the percentage of mussels with a given intensity of haemocytic neoplasia in the sample of mussels examined, site indicates the tissue, organ, or part of the mussel in which the neoplastic haemocytes were found, and locality denotes the geographic area of collection of the mussels.

DIAGNOSIS OF HAEMOCYTIC NEOPLASIA IN MUSSELS

Diagnosis was made by histopathological examinations of fresh or stained haemolymph preparations or of histological tissue sections. Neoplastic haemocytes have abnormal nucleus to cytoplasm ratios (nucleus 6 to 8 μm in 8 to 10 μm cell) in contrast to normal blood cells (nucleus 2 to 3 μm in 4 to 6 μm cell) (Alderman and Green 1985). In fresh and stained haemolymph preparations, neoplastic haemocytes tend to stay rounded (Fig. 1a and b) and usually do not attach to the surface of the glass slide nor spread as normal haemocytes (Fig. 2). Thus, to maintain neoplastic haemocytes on the glass slides for stained preparations, the slides were preconditioned by adding a film of Poly-L-lysine (Appendix III) to improve the adhesiveness of these cells. To determine the intensity of haemocytic neoplasia in a mussel, the percentage of neoplastic cells in a "random" subsample of 100 to 500 haemocytes on a stained preparation (see Appendix I and II for sampling haemocytes and Appendix III for staining procedures) was calculated or the relative abundance of neoplastic haemocytes was estimated after scanning the entire stained preparation from each mussel. When estimating the relative abundance of neoplastic haemocytes, the intensity was categorized as belonging to one of four possible conditions: 1) <10%; 2) 10 to 40%; 3) 40 to 80% or 4) >80% of the haemocytes being neoplastic. In some investigations, the intensity of haemocytic neoplasia is reported as being light or heavy. Light intensities occurred in mussels with 10 to 40% of the cells being neoplastic and heavy intensities in mussels with greater than 40% of the cells being neoplastic.

Histological tissue sections of mussels were used to confirm the presence and relative intensities of haemocytic neoplasia as determined from stained haemolymph preparations, and to describe the sites of neoplastic haemocyte accumulations within the mussel. Relative intensities were recorded as either light or heavy. Light intensities occurred in mussels with slightly greater than normal numbers of haemocytes of which some were neoplastic. Neoplastic haemocytes were usually first observed in the connective tissue between the tubules of the digestive gland. Heavy intensities occurred in mussels with numerous neoplastic haemocytes in most of the tissues. In histological sections, most haemocytes were rounded. However, the abnormal nucleus to cytoplasm ratios of neoplastic haemocytes was evident and there was some displacement of normal tissues in mussels with heavy intensities (Fig. 3a and b). In contrast, the haemocytes in normal mussels each had a smaller nucleus and normal haemocytes were usually fewer in number (Fig. 4a and b).

DESIGN OF REPORT

This report consists of seven separate investigations as presented below. Each investigation contains an introduction that presents the purpose of the work, and a complete material and methods, and results section. A discussion section that summarises the results and highlights the unique observations in that investigation is also included. A conclusion (comprehensive discussion) that incorporates the results of all investigations is presented at the end of this report.

INVESTIGATION 1

ASSAY OF MORTALITY DURING THE SUMMER AND PREVALENCE OF HAEMOCYTIC NEOPLASIA IN MUSSELS FROM DEPARTURE BAY

INTRODUCTION

In previous studies (Quayle 1978, Heritage 1983), unexplained mortalities were observed in mussels larger than about 4 cm from Departure Bay. The present investigation was designed to document the extent of the mortalities that occurred in caged mussels in Departure Bay during the summer months (late May to mid September) and to monitor the mussels in an attempt to identify any correlation between the mortalities and the prevalence and intensity of haemocytic neoplasia. In addition, the prevalence and intensity of haemocytic neoplasia in wild mussel stocks from Departure Bay were monitored periodically throughout the year.

MATERIALS AND METHODS

Mortalities, and prevalence and intensity of haemocytic neoplasia were closely monitored in six groups of mussels taken from wild stocks in Departure Bay at about two week intervals beginning on 28 May 1986 and ending on 17 September 1986. Each group consisted of 90 mussels from 35 to 50 mm in shell length. Thirty mussels were individually marked with colour coded and numbered queen bee discs (about 2 mm in diameter) that were glued to the shell and haemolymph samples were taken (Appendix I). These mussels were grouped into one pearl net. Two other lots of about 30 mussels each, which were not marked nor bled and served as controls, were placed into two other similar pearl nets. The pearl nets were hung about 1 m below the surface off a floating wharf in Departure Bay at the Pacific Biological Station. Every two or three days, all mussels were examined and dead or moribund individuals were counted and discarded. Every two weeks the haemolymph of surviving marked mussels was resampled. All haemolymph samples were preserved and stained (Appendix III) and the intensity of neoplastic cells determined.

In addition to this experiment, the prevalence and intensity of haemocytic neoplasia in other mussels (35 to 50 mm in shell length) from Departure Bay wild mussel stocks was determined periodically throughout the year as described above.

RESULTS

Repeated sampling of mussel haemolymph was detrimental to the mussels as indicated by mortality rates which were from two to four times higher than those of unbled controls (Table 1). The high mortality (100%) incurred after the first sampling of mussels in group 6 may be attributable to stress induced by the exceptionally warm sea water (23°C) in which the mussels were placed shortly after being sampled. Regardless of when they were collected from the wild population, only 11 of 542 mussels (including controls, Table 1) survived beyond 17 September 1986.

The prevalence of the neoplastic condition at the first sampling of each group of mussels varied between 12% and 25% (Table 1). Most of the variation is probably attributable to the relatively small numbers (24 to 30) of mussels examined at each sampling date. The majority of mussels with neoplasia died after the first sampling. Thus, the prevalence of neoplasia was reduced from 18.0% (29/161) in all groups of mussels that were sampled for the first time to 7.9% (5/63) by the second sampling, and to 0% (0/43), 7.7% (2/26), and 11.1% (1/9) in the subsequent three samplings. Four of the 63 mussels that were bled more than once had an increase in the percentage of neoplastic cells from less than 10% to greater than 80% within the two week interval between repeat sampling. Three other mussels that showed a similar increase in intensity of neoplastic cells did so over four weeks (or two repeat samplings) and one mussel maintained greater than 80% neoplastic cells for two subsequent samplings (at least two weeks). Two other mussels showed a substantial reduction in the percentage of neoplastic cells from greater than 80% to about 10% in the two week intersampling interval, whereas five others had lesser reduction of intensities from between 10 to 40% to less than 10% in the same interval. Thus, the neoplastic condition can develop quickly and some mussels are apparently capable of curing themselves of the condition.

There was no apparent seasonal variation in the prevalence or intensity of neoplasia in mussels from Departure Bay (Table 2). In fact, the highest variation in prevalence of neoplastic mussels was between samples taken in late May of 1986 and those taken in early June of 1987. Thus, there was no

apparent correlation between the high mortalities in the late summer of each year and the prevalence of the neoplastic condition.

DISCUSSION

The high mortality rate (98%) observed during this investigation was similar to that observed by S. Swarbrick and G. Jamieson (pers. comm.) who were investigating mussel culture potential in the same locality. The prevalence of mussels with a heavy intensity of haemocytic neoplasia varied irregularly between 8% and 22% during the year (Table 2) whereas the percentage of mussels (in control, unbled groups) that died during each two week interval increased from about 15% in June and early July to about 32% in August and to about 60% in September (calculated from data in Table 1). Thus, there was no apparent correlation between the prevalence of haemocytic neoplasia and mortality of mussels in Departure Bay.

Seven of the 63 mussels (11.1%) that were bled more than once had reductions in the abundance of neoplastic cells. Remission of haemocytic neoplasia in mussels was observed by Elston et al. (1988a) who reported that 8 of 40 mussels (20%) appeared to be in a state of remission at the termination of their 128 day experiment. The higher percentage of remission noted by Elston et el. (1988a) corresponded with reduced mortalities (12 of 40 or 30% died) incurred during their study. In the present study 98% of the mussels died despite the fact that 86 of the 542 mussels had only been in the experiment for 43 days. These differences in percentage of remission and total mortality between the present investigation and that of Elston et al. (1988a) may be attributable to differences in experimental design. Mussels were held in laboratory tanks at 9 to 12°C during the winter months in the latter study.

INVESTIGATION 2

PREVALENCE OF HAEMOCYTIC NEOPLASIA IN MUSSELS FROM THREE SITES, SIX MONTHS AFTER RECIPROCAL TRANSPLANT STUDIES

INTRODUCTION

This investigation was carried out in conjunction with a study (S. Swarbrick pers. comm.) to examine the influence of the environment (grow-out locality) and potential genetic differences in mussel populations (seed source) on growth and survival. After the mussels had been at the reciprocal transplant localities for 6 months, the Swarbrick study was terminated and subsamples of all mussel groups were examined for haemocytic neoplasia. The prevalence and intensity of haemocytic neoplasia in each group was then compared to their mean survival in hope of finding some correlation between neoplasia and mortality.

MATERIALS AND METHODS

Reciprocal transplants were conducted on April 15, 1986 between three localities in British Columbia: Departure Bay in the Strait of Georgia, Sproat Bay near Bamfield in Barkley Sound, and Booker Lagoon in Queen Charlotte Strait. At each locality, replicate groups of mussels (about 3.5 cm in shell length) from each seed source were suspended on Vexar plastic strips in cages to eliminate predation (S. Swarbrick pers. comm.). When the study was terminated in mid-October 1986, haemolymph of 19 to 29 mussels from each of the seed sources at each of the grow-out localities was collected (Appendix II) and the intensity of haemocytic neoplasia was determined for each mussel.

RESULTS

The prevalence of mussels with haemocytic neoplasia from each of the seed sources after 6 months at reciprocal grow-out localities varied from 10% to 36% (Table 3). The lowest mean prevalence of neoplasia (16%) occurred in mussels at the Departure Bay grow-out locality which had the lowest mean percent survival (8.6%). The highest mean prevalence of neoplasia (29%) occurred in mussels at the Booker Lagoon grow-out locality which had the highest mean percent survival (15.6%). However, there was no apparent correlation between the prevalence of mussels with advanced neoplasia ($\geq$40% intensity) and mean percent survival for each of the seed sources within a grow-out locality, or between localities. For example, mussel seed from Departure Bay grown-out at Bamfield had a low mean percent survival of 3.1%, and 24% of the mussels had advanced neoplasia. The same prevalence of mussels with advanced neoplasia (24%) was observed in the group of mussels (Bamfield seed source and Booker Lagoon grow-out) with the highest mean percent survival (30.1%).

DISCUSSION

The variation in survival and prevalence of neoplasia in mussels from the same seed source and grown at different localities may indicate that the environment has some influence on the development of neoplasia as well as on survival. However, no correlation was found between the prevalence of neoplasia in a group of mussels and the percentage of mussels that died.

INVESTIGATION 3

PREVALENCE OF HAEMOCYTIC NEOPLASIA IN MUSSELS HELD IN PEARL NETS FOR VARYING PERIODS OF TIME AT FIVE LOCALITIES

INTRODUCTION

This investigation was carried out in conjunction with a study designed and conducted by G. Jamieson (pers. comm.) in 1987 to examine rate of growth and percent survival of mussels at five localities in British Columbia. During periodic nondestructive sampling of mussels at each locality additional mussels from unsampled pearl nets were collected and returned to us for examination. The condition of these mussels and the prevalence of haemocytic neoplasia were compared to mean survival for each period at each location.

MATERIALS AND METHODS

During the last week of April, 1987, mussels (from 30 to 35 mm in shell length) were collected from local wild stocks and placed into pearl nets in each of five localities: Brandon Island in Departure Bay, Tzartus Island near Bamfield in Barkley Sound, Booker Lagoon in Queen Charlotte Strait, Indian Arm near Vancouver off Burrard Inlet, and Trevenen Bay in Okeover Arm (G. Jamieson pers. comm.). The groups of mussels used in this investigation to determine the prevalence of haemocytic neoplasia were set up with 50 mussels in each of 6 pearl nets. (The mussels used in the Jamieson study were also placed into 6 pearl nets, but with 100 mussels per net.)

On June 11 to 25, August 14 to 26, and October 9 to 14, 1987 the mussels from each locality in the Jamieson study were examined in a nondestructive manner. At these times, all mussels in one pearl net of the neoplasia study group from each locality (except for the June sample at Indian Arm when a pearl net was not collected) were transported live to the Pacific Biological Station, Nanaimo, British Columbia, for examination within three days of collection. Between February 5 to 12, 1988, all remaining pearl nets used for the neoplasia study at each locality were collected and surviving mussels examined. However, only 2 of the original 150 mussels in three pearl nets from

Booker Lagoon were still alive and were not examined, and all remaining pearl nets at Trevenen Bay were lost prior to February due to vandalism.

In addition to examining the haemolymph of each mussel for the percentage of neoplastic haemocytes (Appendix II and III), shell size, soft tissue wet weight, relative body condition, and reproductive state as determined by a wet mount microscopic examination (100X to 400X magnification) of gonadal tissue in the mantle of each mussel were recorded.

RESULTS

Although the prevalence of haemocytic neoplasia and percent survival varied at all locations (Table 4), there was no apparent correlation between these two factors. For example, the prevalence of neoplasia in three localities (Brandon Island, Tzartus Island, and Trevenen Bay) varied from 0 to 12.9% in June 1987. By late August 1987, the mortalities during the intervening six weeks at each of these localities was about the same (34.6 to 31.8%). Also, despite the high percent survival of mussels in Indian Arm (76.6%), the prevalence of neoplasia at this locality in early October 1987 was at least three times that in two other localities (Trevensen Bay, and Tzartus Island) that experienced considerably poorer survival (24.2 and 26.7%, respectively) by October (Table 4). An exception occurred at Booker Lagoon where percent survival was very low and prevalence of neoplasia high (22.6% in June to 90% in August). Unfortunately, the sample sizes were too small to interpret the significance of this observation.

The relative condition of the mussels, as indicated by the percentage of mussels with body tissues that filled the shell cavity, varied inconsistently between locality and sampling date (Table 4). There was also no apparent correlation between the mussels in apparently good condition and the sex, percent spawning, or prevalence of neoplasia. However, it was noted that most mussels in the advanced stages of the neoplastic condition (i.e., $\geq$40% of the haemocytes with neoplastic characteristics) tended to have thin, watery tissues and showed no gonadal development (Fig. 3b).

Spawning mussels were found in at least some localities at each sampling period (Table 4). The June 1987 sample from all localities generally had the highest percentage of mussels in spawning condition although up to 62% of the mussels were spawning in some localities in August 1987 and February 1988.

However, for all localities, the samples with the least spawning activity (i.e., lowest percentage of mussels with numerous fully developed gametes in mantle tissues and highest percentage of mussels with gonadal atrophy such that the sex was not identifiable) were collected in early October 1987.

DISCUSSION

As in Investigation 2, there was no consistent correlation found between the prevalence of haemocytic neoplasia and mortality in the mussels at the five localities. Although the mussels about 4 cm in shell length or greater suffered high mortalities at 4 of the 5 localities examined, there were numerous small mussels present at all localities. Thus, despite the presence of haemocytic neoplasia and high annual mortalities in the larger sized mussels, the actual abundance of mussels did not seem to be affected.

In general, there was a loss of spawning activity of mussels in the late summer and early fall at most localities. This reduction in spawning activity correlated with the time of year that the majority of mussels died. However, there was no correlation between increased mortalities and the prevalence of neoplasia in the mussels at this time of year. Thus, it is possible that mussels from some localities experience post-spawning senescence with associated death. Neoplasia may be one of the repercussions associated with senescence but does not seem to be a necessary prerequisite of mortality.

INVESTIGATION 4

EFFECT OF TEMPERATURE AND SALINITY ON SURVIVAL AND HAEMOCYTIC NEOPLASIA IN MUSSELS FROM DECEMBER TO MARCH

INTRODUCTION

In Departure Bay, many of the larger mussels (about 4 cm in shell length) die during the late summer after experiencing fluctuations in the water temperature and salinity throughout the spring and summer months. To determine the extreme conditions that the mussels might be exposed to, a record of the surface temperature and salinities accumulated from April to November of 1986 at the Canada Department of Fisheries and Oceans Brandon Island experimental fish culture facility in Departure Bay was obtained (A. Solmie, pers. comm.). From these temperature and salinity profiles (Fig. 5) extremes of about 20°C and 22 °/$_{oo}$ salinity during the summer months were identified and selected for experimental work. During the winter months (December 1986 through March 1987) mussels were exposed to a combination of these conditions and ambient sea water in the laboratory in an attempt to identify if these conditions affect mussel survival and/or the prevalence and intensity of haemocytic neoplasia. In addition, the prevalence of haemocytic neoplasia as detected by the sampling of haemocytes (Appendix II) and by histological examination were compared.

MATERIALS AND METHODS

On the 27 November 1986, 1,200 mussels from Departure Bay between 3.5 and 5.0 cm in shell length were randomly divided into 20 groups. Each group of 60 mussels was placed on a circular sheet of 6 mm mesh stiff plastic Vexar and suspended about 10 cm from the bottom and above the outflow stem pipe of a 50 L pot tank. The mussels were placed on the Vexar to allow them to be easily removed from the tank for periodic superficial examination without damaging the byssal attachments. About 2.5 L/min ambient sea water (9.5°C and 31 °/$_{oo}$ salinity) was supplied to the top of each tank. Twice a week, the water flow was diverted for about 2 hr and a suspension of cultured phytoplankton (Isochrysis galbana (T-iso clone) or Chaetoceros calcitrans) or diatoms scrapped from other seawater tanks in the laboratory was added as a source of

food for the mussels. After 12 days of acclimation and on 9 December 1986 (day 0), all moribund mussels were removed and the number of mussels in each tank was adjusted to about 50. Within 6 hr, the water temperature and salinity in 4 groups of 5 tanks per group was adjusted to one of four conditions: Group A, ambient temperature and salinity, 9.0°C and 31 °/₀₀; Group B, average warm temperature and lowest salinity recorded in surface water of Departure Bay between April through November 1986 (Fig. 5), 19.5°C and 22 °/₀₀; Group C, ambient temperature and low salinity, 9.0°C and 22 °/₀₀; and Group D, warm temperature and ambient salinity, 19.5°C and 31 °/₀₀. During the experiment (107 days, to 26 March, 1987) the temperature and salinity varied less then 4.5°C and 4 °/₀₀ respectively in each group of tanks with the greatest variation occurring in the warmer tanks at low salinity (Table 6).

Every second or third day, all mussels were superficially examined after lifting the vexar with attached mussels out of each tank. All dead mussels were counted and removed. On day 21 (Dec. 30), day 50 (Jan. 28), day 80 (Feb. 27), and day 107 (March 26), about 25 mussels were removed consecutively from the last tanks in each of the four groups. Haemocyte preparations (Appendix II) and histological tissue sections (routine procedure of fixing shucked mussels in Davidson's fixative, imbedding tissues in paraffin, and staining sections with haematoxylin and eosin) were made and examined for each mussel. Mussels from the first three tanks of each group were not sampled until the experiment was terminated and these mussels were used to monitor survival in each group throughout the experiment. When the experiment was terminated on day 107 (26 March 1987), all remaining mussels were examined over a period of 11 days. The shell lengths and soft tissue wet weights were measured. The tissue abundance and condition was classified into one of three categories: 1) tissue filling shell, 2) tissue not quite filling shell therefore mussel slightly emaciated, and 3) tissues translucent and watery and not filling shell. Also, the gonad was examined microscopically to identify the sex and estimate the abundance of mature gametes. The mortality rate, condition of surviving mussels, and prevalence of neoplasia in each group of mussels as well as the detection of neoplasia by haemocyte and histological examination was compared.

RESULTS

During the 107 day incubation period, 32% of the 611 mussels in the unsampled tanks died (Table 5). The mussels in group D held at the warm temperature (19°C) and ambient salinity

(31 °/$_{oo}$) experienced a significantly greater total mortality (64%) than the other three groups (Chi-square test, $P<0.05$). The total mortalities in the other three groups were statistically similar (Chi-square test, $P>0.05$) and ranged from about 26% in group B at the warm temperature (19.5°C) and low salinity (22 °/$_{oo}$) to about 18.5% in the remaining two groups, A and C, both at ambient temperature (9.0°C) but different salinities of ambient (31 °/$_{oo}$) and low (22 °/$_{oo}$), respectively.

Throughout the experiment, the mussels showed no appreciable growth. Thus, when the experiment was terminated, the mean shell lengths of the mussels in all 4 groups were not significantly different (Table 5, T-test, $P>0.05$). However, the mussels had been feeding on the phytoplankton provided as indicated by the gut contents of mussels examined histologically and by the high percentage of mussels that were not translucent and watery after 119 days in the tanks. Nevertheless, the mean wet weight of soft tissues (meat) was significantly reduced in the mussels of group D that suffered the highest mortality (T test, $P<0.001$) and was significantly greater in the mussels of group C at ambient temperature but low salinity (T test, $P<0.001$). This same relationship prevailed for the tissue condition (Table 5). The percentage of gravid mussels in the two groups incubated at ambient temperatures was significantly greater than those at the warm temperatures (Chi-square test, $P<0.05$, Table 5). Also, the mussels of group D that experienced the highest mortalities, had the most mussels with undeveloped gonads (39%) and none were gravid.

The prevalence of neoplasia in mussels examined throughout the experiment was low with only 40 of 385 mussels (10.4%) having at least a few neoplastic haemocytes (Table 6). Of these 40 mussels only 23, or 6% of all mussels examined, had intensities of neoplastic haemocytes which were probably heavy enough to be lethal for the mussel. The mussels of group D with the highest mortality had the lowest prevalence of neoplasia (Table 6).

In addition to the mussels with neoplasia, 53 (8.7%) had an abnormally high number of normal-looking haemocytes in the connective tissue, between the digestive gland tubules, and/or in the gills (Table 6). These cells were in diffuse patches (Fig. 6a) or tightly packed in whorl-like patterns (Fig. 6b). Dense patches or whorls of normal haemocytes were observed between the epithelial cells of the intestinal wall of some mussels and apparently undergoing the process of diapedesis (Fig 7a and b). In all cases, the cause(s) of these phenomena was not evident.

The 385 mussels sampled on days 21, 50, 80, and 107 were examined for haemocytic neoplasia using both haemocyte preparations (Appendices II and III) and histological sections. The examination of histological sections gave more reliable

results with about twice as many light intensities of haemocytic neoplasia being detected using this technique (Table 7). Also, 21 of the 385 haemocyte preparations (5.5%) could not be read due either to insufficient haemocytes in the preparations or to poor staining: features which were variable for each preparation. Nevertheless, the prevalence of neoplasia (including both light and heavy intensities as presented in Table 7) detected by the two techniques was not significantly different (Chi-square test, $P>0.05$).

DISCUSSION

The prevalence of haemocytic neoplasia observed in this investigation did not correlate with the number of mussels that died. In group D, the majority of the neoplastic mussels may have died but many of the mortalities must be attributed to other unknown causes. If neoplasia was the only cause of mussel mortality in group D, at any point in time (sampling day), mussels with developing neoplasia should have been present. In fact, only 4 of 89 mussels (3.4%) examined from group D had any evidence of neoplastic cells (Table 6). This prevalence of neoplasia was greatly reduced from the mean prevalence of 12.5% observed in the other three groups. In the other groups (A,B,and C), the mortalities could be attributed to neoplasia but all mussels with neoplasia could not have died between sampling dates because the mortalities would have been higher than observed.

Although there was no apparent correlation between the prevalence of neoplasia and mortality, the data suggest that mussels incubated at warm temperatures and ambient salinity (group D) have poorer survival and reduced prevalence of neoplasia than mussels incubated at the other conditions tested.

The prevalence of haemocytic neoplasia as determined by examining histological sections and haemocyte preparations from the same mussels gave similar results. This comparison confirmed the reliability of haemocyte preparations for detecting haemocytic neoplasia as indicated by other workers (Farley et al. 1986, Elston et al. 1988a). Thus, the use of haemocyte preparations to evaluate prevalence and intensity of neoplasia in mussels, especially for sequential samples of the same mussel (as utilized in Investigations 1), is valid and representative of actual conditions.

INVESTIGATION 5

EFFECT OF TEMPERATURE ON SURVIVAL AND HAEMOCYTIC NEOPLASIA IN SMALL AND LARGE MUSSELS FROM APRIL TO NOVEMBER

INTRODUCTION

In Investigation 4, the high mortalities experienced by large mussels in Departure Bay during the late summer and fall could not be reproduced in the laboratory during the winter months by experimentally exposing the mussels to warm temperatures and low salinities. The converse of Investigation 4 would be an attempt to circumvent the summer mortalities by experimentally holding mussels at a cool temperature throughout the summer and fall. Thus, the purpose of this investigation was to determine if the summer mortality syndrome and occurrence of haemocytic neoplasia could be prevented or reduced by maintaining mussels at a relatively constant cool temperature (about 11°C) from April to November. Small mussels, which usually do not experience the summer mortality syndrome, were also maintained under similar conditions for comparison.

MATERIAL AND METHODS

On 7 May 1987, two groups of about 225 large mussels (3.5 to 5.0 cm in shell length) and two groups of about 225 small mussels (1.0 to 1.5 cm in shell length) were collected from Departure Bay. Each group of mussels were placed on a 58 by 42 cm sheet of stiff plastic Vexar (6 mm mesh) that was suspended above the outflow stem pipe and about 15 cm from the bottom of a rectangular 110 L flow through tank. The Vexar was utilized in this investigation for the same reasons it was used in Investigation 4. The four tanks were each supplied with 4.5 L/min ambient sea water (13°C and 29 °/$_{\infty}$ salinity). Every Monday, Wednesday, and Friday, the water flow was diverted for about 4 hr and 1 L of about 3 to 4 million cultured cells/ml of Isochrysis galbana (T-iso clone) or occasionally Chaetoceros calcitrans was added to each tank.

After 11 days of acclimation and on 18 May 1987 (day 0), the number of mussels in each tank was adjusted to about 200. The water supply to one tank of large mussels and to one tank of

small mussels was adjusted to about 11°C and 29 °/$_{oo}$ salinity. Throughout the experiment (175 days) the water temperature and salinity in these two tanks was measured three times a week and if necessary was readjusted to these designated values, which are representative of winter conditions. The actual fluctuations in temperature and salinity (Figs. 8 and 9, respectively) recorded in these two tanks was the same (both on same water supply). The water supply to the other two tanks, also one each of large and small mussels, was adjusted every Monday, Wednesday, and Friday to approximate surface water temperature and salinity measured on those days in Departure Bay off the wharf at the Pacific Biological Station. The temperature and salinity profiles in the two tanks (both on same water supply) and at the surface of Departure Bay are presented in Figs. 8 and 9, respectively.

At the time of monitoring the water conditions in the tanks and prior to adding the phytoplankton, all mussels were superficially examined and dead mussels recorded and removed. When the experiment was terminated on 9 November 1987 (day 175), the shell length of all surviving small mussels was measured and a random sample of 20 mussels from each group was preserved in Davidson's fixative and examined histologically for neoplastic haemocytes following standard procedures and staining with haematoxylin and eosin. All surviving large mussels were measured (shell length and soft tissue wet weight), the tissue condition classified into one of three categories as in Investigation 4, the sex and maturity of the gonad determined after microscopic examination (250 X magnification), and haemocyte preparations made (Appendix II). In addition, a random sample of 20 mussels from each group was preserved and examined histologically as for the small mussels.

RESULTS

In the two tanks maintained to approximate winter conditions, the water temperature was cool but fluctuated between 10 and 13°C for the first 108 days and dropped to between 10 and 8°C for the last 35 days (Fig. 8). However, on day 110 a high temperature of 14°C, and on day 134 a low of 4°C were accidentally experienced. The water temperature in the other two tanks ranged seasonally from 9.5 to 21°C and fairly closely approximated the surface water temperatures of Departure Bay (Fig. 8). The salinity in both sets of tanks was highly variable especially during the first 100 days of incubation (Fig. 9). However, the salinity in both sets of tanks was usually within 2 °/$_{oo}$ of the salinity of the surface water in Departure Bay.

When the experiment was terminated, the accumulative mortalities (Table 8) of both sizes of mussels held at the two water temperatures were significantly different (Chi-square test, $P<0.05$). The greatest mortalities in both sizes of mussels occurred in the groups incubated at the ambient and thus warmer temperatures (Fig. 10). However, more mussels in the large size groups died than in the small size groups. In fact, the percentage of mussels that died in the group of large mussels held at the cooler temperature was not significantly different from the percentage of mussels that died in the group of small mussels held at the warmer ambient temperatures (Chi-square test, $P>0.05$).

The total number of small mussels accounted for at the termination of the experiment was reduce by about 13% from the number of mussels placed in the tanks when the experiment was initiated. A few of these small mussels may have escaped from the tank by crawling out the stem pipe. However, it is more likely that most died and totally disintegrated before the empty shells were found. If all small mussels that were unaccounted for had died, then the cumulative mortalities for the small mussels at the cooler temperature would not have been statistically different from those of the larger mussels also at this temperature, but the other comparative relationships would not have changed.

None of the mussels grew during the 186 day incubation period. Thus, at the termination of the experiment, the shell lengths and soft tissue wet weights (Table 8) were not significantly different within each size group held in the two different water conditions (T test, $P>0.05$). The tissue condition of the two groups of small mussels was not examined. However, in the two groups of large mussels, 72 of the 76 survivors showed slight tissue emaciation. Of these remaining four, three were extremely emaciated with translucent and watery tissues and only one mussel had sufficient tissue to fill the shell. None of the 76 large mussels were gravid and gametes were not evident in 65 of them.

Haemocytic neoplasia was observed in about 33% of the large mussels and in over twice as many (70%) small mussels (Table 8). The prevalence and heavy intensity of neoplastic haemocytes in the two groups of large mussels maintained in the two water conditions were not significantly different (Chi-square test, $P>0.05$). However, there was a significantly higher prevalence of neoplasia in the small mussels held at the cooler temperature than in those held at the warmer ambient temperature (Chi-square test, $P<0.05$).

Fourteen of the 40 large mussels that were examined histologically had diffuse patches (Fig. 6a) or tightly packed whorl-like patterns (Fig. 6b) of normal-looking haemocytes in the

connective tissue and/or between the digestive gland tubules. Four of these mussels had light intensities of haemocytic neoplasia. In one mussel, the whorls of haemocytes incorporated the neoplastic haemocytes (Fig. 11a) and in the other three mussels, neoplastic haemocytes were observed around the whorls of haemocytes but not incorporated within them (Fig. 11b). In three other mussels without haemocytic neoplasia, the whorls of normal haemocytes surrounded some necrotic cells as well as cells with eosinophilic inclusions to form cyst-like structures (Fig. 12). These cyst-like structures were equally represented in both experimental groups of large mussels and in all cases the cause of the phenomenon was not evident. Insufficient tissue was present in the histological sections of the small mussels to determine if they were also afflicted with this condition.

DISCUSSION

The large mussels maintained at warm ambient (summer) temperatures experienced mortalities approaching those observed for mussels held in the surface waters of Departure Bay (Investigation 1). Although the summer mortality syndrome was significantly reduced by maintaining mussels at a relatively constant cool temperature (about 11°C) from April to November, the percentage of large mussels that died at the cool temperatures was high (73.5%). Also, the percentage of large mussels at the cool temperatures that died was about 10% greater than observed for similar sized mussels held at a warm temperature and high salinity during the winter months (Investigation 4). Thus, the summer mortality syndrome was still detectable during the summer season despite the environmental manipulations achieved in the laboratory. It is possible that the mussels used in this investigation (5) were already predisposed to undergo the summer mortality prior to setting up the experiment in April and holding some of the mussels at cooler temperatures merely slowed down the process.

A complicating factor for the laboratory investigations is the inadequacies of the laboratory system for mussel culture as indicated by the lack of growth and relatively poor survival among the small mussels. Nevertheless, the comparison in survival of mussels between studies conducted during the summer and winter months (Investigations 4 and 5) would be valid because both experiments were conducted in the laboratory under similar maintenance regimens.

Haemocytic neoplasia was not prevented nor reduced in prevalence or intensity by maintaining large mussels at a

relatively constant cool temperature (about 11°C) from April to November. Consequently, the prevalence and intensity of haemocytic neoplasia in the large mussels held at both temperatures were similar, although mussels held at the warmer temperature experienced greater mortalities. Thus, within the two groups of large mussels used in this investigation, there is no apparent correlation between mortalities and neoplasia. However, the prevalence of haemocytic neoplasia in these mussels was about three times as great as observed in the mussels of Investigation 4, which experienced reduced mortalities. Thus, the results from these two investigations (4 and 5) suggest a pattern of increased prevalence and intensity of neoplasia with resulting increases in mortality. This pattern is in direct contradiction with results from the small mussels in Investigation 5 where the highest prevalence of neoplasia recorded to date occurred in the group of mussels with the lowest mortalities. This lack of overall consistency in correlations between neoplasia and mortality again suggests that neoplasia is not the main cause of the mortalities.

The histological observations of the clusters of normal-looking haemocytes (Figs. 6a and b, 11a and b, and 12) are discussed in the "Conclusions" section of this report.

INVESTIGATION 6

SYRINGE PASSAGE OF HAEMOCYTIC NEOPLASIA IN MUSSELS

INTRODUCTION

In an attempt to investigate the infectious nature and pathogenicity of haemocytic neoplasia, mussels from Departure Bay were inoculated with neoplastic haemocytes. The rate of mortality and prevalence of neoplasia in the inoculated mussels (challenged) were compared to these parameters in an equivalent group of mussels inoculated with saline (controls).

MATERIALS AND METHODS

On 13 February 1987, about 150 mussels between 4.0 and 5.5 cm in shell length were collected from Departure Bay and placed in a 200 L tank supplied with flow-through ambient seawater (8.5°C, 31 °/$_{oo}$ salinity). On 16 February 1987, about 50 mussels were examined for haemocytic neoplasia. Neoplastic haemocytes were collected aseptically from 5 of the heavily affected mussels. The cells were pooled and washed three time in autoclaved seawater with antibiotics (100 IU penicillin, 100 µg streptomycin, and 0.25 µg Fungizone^{R} per ml (GIBCO, Grand Island, NY 14072)) followed by centrifugation (2000 g at 5 to 10°C for 10 min) after each wash. The washed cells were resuspended in autoclaved seawater with antibiotics to give 3.8 X 10^{6} neoplastic haemocytes per ml. Each of 50 mussels was inoculated with 0.05 ml of this cell suspension (about 1.9 X 10^{5} neoplastic cells per mussel) in the region of the foot retractor mussel using a 23 gauge 3/4 inch (1.9 cm) needle inserted about 5 mm into the tissue between the valves immediately posterior to the hinge. Inoculated mussels (challenged) were placed onto a circular sheet of stiff plastic Vexar (6 mm mesh) and suspended about 10 cm from the bottom and above the outflow stem pipe of a 50 L pot tank. An additional 50 mussels were inoculated in a similar manner with autoclaved seawater and antibiotics but no neoplastic haemocytes to serve as controls. As for the previous group of mussels, these mussels (controls) were placed on Vexar in a separate 50 L pot tank.

Both tanks were each supplied with 4.5 L/min ambient sea water that varied seasonally from 8.0°C and 31 °/$_{oo}$ salinity to 12.5°C and 29 °/$_{oo}$ salinity. Every Monday, Wednesday, and Friday, the water flow was diverted for about 4 hr and 0.5 L of about 3 to 4 million cultured cells/ml of Isochrysis galbana (T-iso clone) was added to each tank as a source of food for the mussels. Prior to adding the phytoplankton, all mussels were superficially examined and dead mussels recorded and removed.

On 19 August 1987 (day 185) when the experiment was terminated, all surviving mussels were measured (shell length and soft tissue wet weight), and tissue condition was classified into one of three categories as in Investigation 4. Soft tissues were preserved in Davidson's fixative and examined histologically for neoplastic haemocytes following standard procedures and staining with haematoxylin and eosin.

RESULTS

During the 185 day incubation period, 73.9% of the challenged mussels and 75.5% of the control group died (Fig. 13). The rate of mortality was fairly consistent throughout the incubation period and was similar for both groups. However, there was a marked difference in the prevalence and intensity of haemocytic neoplasia in the surviving mussels of each group (Table 9). Nine of 12 challenged mussels had neoplastic haemocytes with heavy intensities in 6 of these. However, only 2 of the 13 surviving control mussels had neoplastic haemocytes, one at each level of intensity.

Tightly packed whorl-like patterns of apparently normal haemocytes (Fig. 6b) were observed in 9 of the 12 surviving mussels that had been challenged and in 7 of the 13 surviving mussels in the control group. Four of the 9 challenged mussels and 1 of the 7 control mussels with these whorls also had haemocytic neoplasia. However, in only one mussel (challenged group) were neoplastic haemocytes incorporated into the whorls (Fig. 11a). In 4 of the mussels (3 challenged and 1 control), these whorls surrounded or encapsulated some necrotic cells as well as cells that contained eosinophilic inclusions (Fig. 12).

There was no apparent growth in the mussels from either group throughout the 185 day incubation period. Thus, at the termination of the experiment, there was no significant difference in the shell length and meat wet weight (Table 9) between the two groups of mussels (T test, $P>0.05$). The tissue condition of all mussels ranged from tissues that filled the

shell to slightly emaciated. However, there were no apparent differences in tissue condition between the two groups.

DISCUSSION

The mussels challenged with neoplastic haemocytes had a greater prevalence and intensity of haemocytic neoplasia than the controls suggesting that this disease was transmitted via syringe inoculation. These results support those of Elston et al. (1988b), who also found that haemocytic neoplasia could be transmitted allogeneically with intact whole cells.

The mussels used in this investigation (6) came from the Departure Bay mussel population which is known to have haemocytic neoplasia. Thus, the observation of a few control mussels with haemocytic neoplasia was not surprising. Also, similar sized mussels from Departure Bay, which were held in the laboratory during previous investigations (4 and 5 in this report), experienced mortalities without having been challenged by inoculation. Thus, the mortalities that occurred among the control group of mussels during this investigation (6) were expected.

If the transmitted haemocytic neoplasia had been highly pathogenic, the mortalities in the challenged mussels (to which haemocytic neoplasia was shown to be transmitted) should have been greater than for the controls. However, there was no significant difference in mortalities between the challenged and control mussels (Chi-square test, $P<0.05$) suggesting negligible pathogenicity. This apparent lack of pathogenicity is contradictory to the findings of Elston et al. (1988b). This discrepancy may be attributable to the differences in the stocks of mussels that were challenged. Elston et al. (1988b) utilized mussel stocks from California that were prescreened to be free of haemocytic neoplasia. Thus, these mussels may have been more susceptible to the disease then the mussels from an endemic area (Departure Bay). Also the inoculum size used by Elston et al. (1988b) was about 13 times greater than the inoculum used in this investigation. Although there may be some pathogenicity associated with this disease, the affect that this disease has on the mortality of mussel stocks from endemic areas is apparently marginal.

The histological observations of the clusters of normal-looking haemocytes (Figs. 6b, 11a, and 12) are discussed in the "Conclusions" section of this report.

INVESTIGATION 7

DNA CONTENT OF HAEMOCYTES FROM MUSSELS WITH HAEMOCYTIC NEOPLASIA

INTRODUCTION

In order to gain some insight into the nature of the haemocytic neoplasia, the DNA content of haemocytes from normal mussels and mussels with heavy intensities of haemocytic neoplasia was analyzed. If the analysis could clearly discriminate between the normal and neoplastic mussels, it was hoped that this analysis could be used as a less subjective and possibly faster means for determining the intensity of this disease in individual mussels.

This investigation was conducted in conjunction with Dr. W.S. Fisher at the University of Maryland Horn Point Environmental Laboratories (current address, University of Texas Medical Branch, Marine Biomedical Institute, Galevston TX).

MATERIALS AND METHODS

Approximately 0.5 ml of haemolymph was aspirated separately from the area of the adductor muscle of each of 70 mussels from Departure Bay using 3 ml syringes equipped with 20 guage, 1 inch (2.5 cm) needles and containing 0.5 ml of autoclaved seawater and 2% formalin. The fixed cells were sent to Dr. Fisher who treated them with RNAse and then stained them with propidium iodide, a nucleic acid-specific stain (Allen 1983). Fluorescence intensity was measured using a flow cytometer constructed from a fluorescent microscope (Shapiro 1985). Fluorescent intensity of individual cells (which reflects DNA content) was recorded and their distribution examined relative to fluorescent latex bead standards (Fisher and Ford 1988).

RESULTS

Of the 70 mussels that were sampled, the DNA content of seven normal mussels and two mussels with heavy intensities of haemocytic neoplasia were measured. The haemolymph collected from these nine mussels mainly contained cells usually found in the vascular system except for the occurrence of sperm in a few of the gravid males. In addition, well stained haemocyte preparations (Appendix III) and histological sections were available for these mussels. The amount of DNA in the haemocytes from one of the neoplastic mussels (Fig. 14a) with a mean relative fluorescence value of 94.8 was about 1.5 times greater than the normal mussels (Fig. 14b) which had a mean relative fluorescence value ($n = 7$) of 67.0 ± 6.2. The majority of the neoplastic haemocytes from this mussel had multilobed nuclei (Fig. 1a) and the few unlobed nuclei contained at least two nucleoli. Histological sections through this mussel indicated that all sinuses had been occluded with neoplastic haemocytes and large numbers of these abnormal cells had infiltrated all organs. The other mussel with haemocytic neoplasia had a DNA profile and mean relative fluorescence value similar to normal mussels (Fig. 14b). No multilobed nuclei were observed in the neoplastic haemocytes of this mussel (Fig. 1b), but many nuclei had at least two nucleoli. Although about 99% of the haemocytes in this mussel were neoplastic, vascular sinuses were not entirely occluded with neoplastic haemocytes and they occurred mainly in the mantle, gills, and between the tubules of the digestive gland.

DISCUSSION

It is possible to reduce some of the variability observed in the distribution of the haemocyte DNA peaks by separating nuclear DNA from the mitochondrial DNA, which occurs in the cytoplasm (Fulwyler 1979). Nevertheless, the results from the crude preparations utilized in this investigation corroborate the results of other workers. Elston and Kent (1988) indicated that with haemocytic neoplasia in mussels, cell division of haemocyte populations containing normal increments of DNA is first amplified as the disease process begins. The differences in DNA content of the two mussels with haemocytic neoplasia measured during this investigation may relate to the observation that they were at different stages of the disease, with the most advance case occurring in the mussel with the increased DNA peak.

The wide distribution of the DNA peaks, that may partially account for the overlap between the normal mussels and one of the neoplastic mussels, indicates that this technique, as we applied it, would not be useful for evaluating intensity of disease in individual mussels. An additional difficulty of aspirating cells other than haemocytes during the collection process was also encountered. Haploid sperm collected from mature male mussels was easily differentiated from diploid blood cells and could even be used as an internal standard. However, in some of the normal mussels (not included in the analysis) the haemolymph samples were contaminated with many other cells including clumps of ciliated epithelial cells and diatoms that were probably aspirated from the mantle cavity or intestinal tract. This contamination probably resulted from our attempts to collect the optimum number of haemocytes that were required for the analysis from mussels that were comparatively small in size. Unless the analysis can be preformed on small samples of haemocytes and unless haemolymph samples are further processed to remove the cytoplasmic mitochondrial DNA, this technique will not be useful as a method for measuring intensity of haemocytic neoplasia in blue mussels from British Columbia.

CONCLUSIONS

Haemocytic neoplasia has been reported from mussels in many parts of the world including Europe, where the prevalence is usually less than 4% (Lowe and Moore 1978, Green and Alderman 1983, Alderman and Green 1985, Rasmussen 1986). On the western coast of North America prevalences of haemocytic neoplasia are much higher and can exceed 40% in some localities (Mix 1983; Cosson-Mannevy et al. 1984; Elston et al. 1988a). In conjunction with this high prevalence of haemocytic neoplasia, mussels at several localities in British Columbia and Washington experience high mortalities after their first spawning (Quayle 1978; Heritage 1983; Emmett 1984; Skidmore and Chew 1985; Emmett et al. 1987; Swarbrick et al. submitted). Recently Elston et al. (1988a, b) demonstrated that haemocytic neoplasia in mussels from Puget Sound, Washington is progressive, lethal, and can be transmitted by cohabitation. Thus, circumstantial evidence suggested that haemocytic neoplasia may be the cause of the high mussel mortalities in British Columbia.

In the present study, the results of the first 6 investigations that concerned prevalence of neoplasia and incidence of mortality indicated that these two parameters were not interrelated. As indicated by Elston et al. (1988a, b), the present investigations confirmed that haemocytic neoplasia is a lethal and infectious disease of mussels. Also haemocytic neoplasia was observed at all localities in British Columbia where mussels were examined. However, there was no consistent positive correlation between mussel mortality and the prevalence or severity of this disease. At each of the localities at which high annual (summer) mussel mortalities were observed, there was no apparent adverse effect on recruitment and numerous small mussels were consistently present. Thus, haemocytic neoplasia has little apparent impact on mussel abundance in British Columbia. In fact, this high mussel recruitment makes mussels one of the major fouling pests of salmon net-pens in British Columbia.

Throughout the present study, no etiological agent was observed that could account for the high mortalities among the mussels over 3 cm in shell length. Possibly as indicated by Emmett et al. (1987) the high mortalities are related to reproductive stress resulting from a reproductive strategy that seems to emphasize high reproductive output at an early age and small size compared to blue mussels from other parts of the world. The other possibility is that the blue mussel in British Columbia and Washington belong to a previously unrecognized distinct species or variety. Recently, McDonald and Koehn (1988) indicated that these mussels are not Mytilus edulis as previously suspected but a more northerly species Mytilus trossulus,

although this latter species is not considered valid by some malacologists (Bernard 1983). Nevertheless, the blue mussels in British Columbia and Washington seem to have a different life cycle from M. edulis in other parts of the world and may be characterized as undergoing reproduction followed by senescence at an earlier age and smaller body size.

The high mortality rate among mussels that are just reaching market size (about 5 cm in shell length) may hinder the development of a mussel culture industry in some British Columbia localities. However, mussels at a few localities, such as Indian Arm (Investigation 3), have substantially higher survival and such localities would be better suited for mussel culture (G. Jamieson pers. comm.). It may also be possible to culture mussels at the localities that experience high summer mortality by modifying culture techniques. During Investigation 5, mussel mortality was significantly reduced by holding mussels at cooler temperatures during the summer months. If mussels were held in the cooler waters at greater depths during certain times of the year (as yet to be precisely determined), high mortalities may be avoided. Another approach would be to create a market for a smaller sized product. This approach would make the vast stocks of our fast growing, small sized mussels an immediate resource.

The patches or whorls of haemocytes observed in the connective tissues of the mussels (Fig. 6a and b) probably consist of a normal cellular response (i.e. inflammatory response) to a stimulant(s). In most mussels, especially those without haemocytic neoplasia, the cause was not evident. Under normal circumstances whorls of normal haemocytes probably leave the mussel by diapedesis (Fig. 7a and b). These idiopathic patches of inflammation apparently also occurred in mussels with haemocytic neoplasia as observed in mussels where the whorls of normal haemocytes did not incorporate neoplastic cells (Fig. 11b). However, in a few neoplastic mussels these whorls incorporated neoplastic cells (Fig. 11a) suggestive of remission as described by Elston et al. (1988a). Mussels containing whorls with necrotic cells and cells with eosinophilic inclusions (Fig. 12) may represent individuals that had successfully destroyed neoplastic cells. However, it is also possible that these lesions are manifestations of other diseases caused by unrecognized invaders such as a virus.

These inflammatory lesions (Fig. 6, 7, 11, and 12) have been observed in mussels collected directly from the field (Bower, unpublished data). However, information on the prevalence of these lesions is only available for the groups of mussels that were used in the laboratory experiments and examined histologically (Investigations 4 to 6). The prevalence varied from 9% in mussels exposed to various temperatures during the winter months (Investigation 4), and 35% in the mussels exposed to various temperatures during the summer months (Investigation

5), to 64% in syringe inoculated mussels held at ambient temperatures (seasonal change form 8.0 to 12.5°C) from mid-February to mid-August (Investigation 6). There was no apparent difference in the prevalences of these inflammatory lesions among the various groups of mussels within each investigation. However, the high prevalence of these lesions in the inoculated group (Investigation 6) suggests that the lesions may be attributable to a nonspecific cellular defence response of the mussel to the inoculations.

ACKNOWLEDGEMENTS

I wish to thank Bruce Leighton and Gary Meyer for their excellent technical assistance. Without their help, the magnitude of this study would not have been possible. Drs. L. Margolis and G. Jamieson provided valuable comments on earlier drafts.

REFERENCES

Alderman, D. J. and M. Green. 1985. Haematopoietic neoplasm in the blue mussel. In: C. J. Sindermann [ed.]. Fiches d'identification des maladies et parasites des poissons, crustacés et mollusques. Conseil International pour l'exploration de la mer. Fische No. 11, 4 p.

Allen, S. K. Jr. 1983. Flow cytometry: assaying experimental polyploid fish and shellfish. Aquaculture 33: 317-328.

Bernard, F. R. 1983. Catalogue of the living Bivalvia of the eastern Pacific Ocean: Bering Strait to Cape Horn. Can. Spec. Publ. Fish. Aquat. Sci. 61: 102p.

Cosson-Mannevy, M. A., C. S. Wong, and W. J. Cretney. 1984. Putative neoplastic disorders in mussels (Mytilus edulis) from southern Vancouver Island waters, British Columbia. J. Invert. Pathol. 44: 151-160.

Elston, R. A. and M. L. Kent. 1988. Biology of the hemic neoplasia in the bay mussel, Mytilus edulis. Third Internat. Colloq. Pathol. Marine Aquacul. 2-6 Oct. 88, Gloucester Point, VA. (U.S.A.). Abstract pg. 85.

Elston, R. A., M. L. Kent, and A. S. Drum. 1988a. Progression, lethality and remission of hemic neoplasia in the bay mussel Mytilus edulis. Dis. Aquat. Org. 4: 135-142.

Elston, R. A., M. L. Kent, and A. S. Drum. 1988b. Transmission of hemic neoplasia in the bay mussel, Mytilus edulis, using whole cells and cell homogenate. Dev. Comp. Immunol. 12: 719-727.

Emmett, B., K. Thompson, and J. D. Popham. 1987. The reproductive and energy storage cycles of two populations of

Mytilus edulis (Linne.) from British Columbia. J. Shellfish Res. 6: 29-36.

Farley, C. A. 1969. Sarcomatoid proliferative disease in a wild population of blue mussels (*Mytilus edulis*). J. Nat. Cancer Inst. 43: 509-516.

Fisher, W. S. and S. E. Ford. 1988. Flow cytometry: a tool for cell research in bivalve pathology. Amer. Fish. Soc. Spec. Publ. 18: 286-291.

Fulwyler, M. J. 1979. Standards for flow cytometry. In Flow Cytometry and Sorting. Edited by M. R. Melamed, P. F. Mullaney, and M. L. Mendelsohn. John Wiley and Sons Inc., New York, pp. 351-358.

Green, M. and D. J. Alderman. 1983. Neoplasia in *Mytilus edulis* from the United Kingdom waters. Aquaculture 30: 1-10.

Heritage, G. D. 1983. A blue mussel (*Mytilus edulis* Linnaeus), culture pilot project in south coastal British Columbia. Fish. Mar. Ser. Tech. Rep. 1174: 27 p.

Lowe, D. M. and M. N. Moore. 1978. Cytology and quantitative cytochemistry of a proliferative atypical hemocytic condition in *Mytilus edulis* (Bivalvia, Mollusca). J. Nat. Cancer Inst. 60: 1455-1459.

Margolis, L., G. W. Esch, J. C. Holmes, A. M. Kuris, and G. A. Schad. 1982. The use of ecological terms in parasitology (Report of an ad hoc committee of the American Society of Parasitologists). J. Parasit. 68: 131-133.

McDonald J. H. and R. K. Koehn. 1988. The mussels *Mytilus galloprovincialis* and *M. trossulus* on the Pacific coast of North America. Mar. Biol. 99: 111-118.

Mix, M. C. 1983. Haemic neoplasm of bay mussels, *Mytilus edulis* L., from Oregon: occurrence, prevalence, seasonality and histopathological progression. J. Fish Dis. 6: 239-248.

Mix, M. C., J. W. Hawkes, and A. K. Sparks. 1979. Observations on the ultrastructure of large cells associated with putative neoplastic disorders of mussels, *Mytilus edulis*, from Yaquina Bay, Oregon. J. Invert. Pathol. 34: 41-56.

Quayle, D. B. 1978. A preliminary report on the possibilities of mussel culture in British Columbia. Fish. Mar. Ser. Tech. Rep. 815: 37 p.

Rasmussen, L. P. D. 1986. Occurrence, prevalence and seasonality of neoplasia in the marine mussel Mytilus edulis from three sites in Denmark. Mar. Biol. 92: 59-64.

Shapiro, H. M. 1985. Practical Flow Cytometery. Alan R. Liss Inc., New York, 259p.

Skidmore, D. and K. K. Chew. 1985. Mussel aquaculture in Puget Sound. Wash. Sea Grant Tech. Rep. 85-4. 57 p.

Table 1. Mortality[1] and prevalence of neoplasia[2] in *Mytilus edulis* (35 to 40 mm shell length) from Departure Bay held in pearl nets from date of first sampling.

		Sample Dates in 1986															
		May 26-28		June 9-11		June 23-24		July 7-8		July 22-23		Aug. 5-6		Aug. 18-19		Sept. 3		Sept. 17
Group	Treatment[3]	Mort.	Neopl.	Mort.	Neopl.	Mort.	Neopl.	Mort.	Neopl.	Mort.	Neopl.	Mort.	Neopl.	Mort.	Neopl.	Mort.	Neopl.	Mort.
1	Bled	0/30	6/27	21/30	0/9	23/30	0/6	28/30	0/2	29/30	0/1	30/30						
	Control 1	0/30	-	5/30	-	7/30	-	14/30	-	20/30	-	25/30	-	26/30	-	27/30	-	30/30
	Control 2	0/31	-	5/31	-	12/31	-	16/31	-	25/31	-	26/31	-	31/31				
2	Bled			0/30	5/30	15/30	1/13	19/30	0/9	20/30	0/9	25/30	0/5	28/30	0/2	30/30		
	Control 1			0/30	-	2/30	-	3/30	-	9/30	-	14/30	-	18/30	-	25/30	-	30/30
	Control 2			0/33	-	5/33	-	9/33	-	15/33	-	24/33	-	27/33	-	29/33	-	33/33
3	Bled					0/30	4/30	13/30	1/16	19/30	0/10	21/30	2/7	29/30	0/1	29/30	0/1	30/30
	Control 1					0/30	-	3/30	-	7/30	-	11/30	-	20/30	-	28/30	-	30/30
	Control 2					0/31	-	5/31	-	13/31	-	19/31	-	24/31	-	29/31	-	31/31
4	Bled							0/30	3/25	16/30	1/12	21/30	0/8	25/30	0/4	28/30	1/2	30/30
	Control 1							0/31	-	7/31	-	18/31	-	22/31	-	25/31	-	28/31
	Control 2							0/30	-	10/30	-	19/30	-	24/30	-	30/30		
5	Bled									0/30	6/24	16/30	2/13	20/30	0/10	26/30	0/4	30/30
	Control 1									0/30	-	6/30	-	14/30	-	28/30	-	30/30
	Control 2									0/30	-	11/30	-	19/30	-	24/30	-	30/30
6	Bled											0/30	5/25	30/30				
	Control 1											0/26	-	5/26	-	18/26	-	21/26
	Control 2											0/30	-	4/30	-	17/30	-	27/30

[1] Recorded as total number of mussels dead by sampling date.
[2] Recorded as number of mussels with >40% of the haemocytes neoplastic/number of mussels sampled. Discrepancies with number alive are due to unreadable preparations.
[3] Mussels of each treatment were held in pearl nets 1 m below the surface. Haemolymph was sampled from bled subgroup as indicated but controls were not bled.

Table 2. Prevalence of haemocytic neoplasia in *Mytilus edulis* (35 to 50 mm shell length) from Departure Bay.

Sample Date	Sample size	Number of mussels with following percentage of neoplastic cells				Prevalence of mussels with neoplasia[1]
		>80%	80-40%	40-10%	<10%	
May 28, 1986	27	5	1	5	16	22%
June 11, 1986	30	4	1	10	15	17%
June 23, 1986	29	3	1	1	24	14%
July 7, 1986	25	3	0	0	22	12%
July 22, 1986	24	5	1	0	18	18%
Aug. 6, 1986	25	3	2	0	20	20%
Oct. 17, 1986	21	3	0	4	14	14%
Oct. 27, 1986	25	4	0	3	18	16%
Jan. 9, 1987	25	3	1	1	17	16%
March 5, 1987	25	3	2	3	17	20%
June 11, 1987	37	1	2	0	34	8%

[1] Mussels with $\geq$40% of cells with neoplastic characteristics on stained preparations of haemocytes were considered as having advanced neoplasia.

Table 3. Prevalence of neoplasia in *Mytilus edulis* (shell lengths 42 to 52 mm) in mid-October 1986, 6 months after reciprocal transplants.

Seed source	Grow-out locality	Sample size	Number of mussels with following percentage of neoplastic cells				Prevalence of mussels with neoplasia[1]	Mean percent survival[2]
			>80%	80-40%	40-10%	<10%		
Departure Bay	Departure Bay	21	3	0	4	14	14%	1.4
Bamfield	Departure Bay	25	5	1	2	17	24%	18.5
Booker Lagoon	Departure Bay	29	2	1	7	19	10%	5.8
Departure Bay	Bamfield	21	4	1	0	16	24%	3.1
Bamfield	Bamfield	19	5	0	4	10	26%	23.5
Booker Lagoon	Bamfield	17	4	1	0	12	29%	4.5
Departure Bay	Booker Lagoon	29	6	2	4	17	28%	4.2
Bamfield	Booker Lagoon	25	4	2	6	13	24%	30.1
Booker Lagoon	Booker Lagoon	28	9	1	6	12	36%	12.5

[1]Mussels with ≥40% of the haemocytes with neoplastic characteristics on stained preparation of body fluids were considered as having advanced neoplasia.

[2]Data from S. Swarbrick (pers. comm.).

Table 4. Size, condition, prevalence of haemocytic neoplasia and percent survival of Mytilus edulis placed in pearl nets during the last week of April 1987 in five localities in British Columbia.

Locality	Date collected	No. of mussels examined	Shell length (mm)[1]	Wet weight of meat (g)[1]	Percentage of mussels with tissues filling shell	Percent male	Percent female	Percent in spawning condition[2]	Prevalence of mussels with neoplasia[3]	Mean percent survival[4]
Brandon Island	June 11/87	41	39.9 ± 4.7	2.26 ± 0.94	80.5	36.6	56.1	48.8	8.1	86.0
Brandon Island	Aug. 14/87	28	43.2 ± 6.6	3.08 ± 1.34	71.4	35.7	46.4	46.4	28.0	50.7
Brandon Island	Oct. 14/87	20	41.0 ± 6.6	2.46 ± 1.36	50.0	20.0	50.0	15.0	25.0	17.0
Brandon Island	Feb. 5/88	25	46.2 ± 6.1	3.19 ± 1.4	36.0	28.0	28.0	32.0	29.2	-
Booker Lagoon	June 17/87	33	39.2 ± 5.1	2.31 ± 0.87	87.9	36.4	57.6	45.5	22.6	73.0
Booker Lagoon	Aug. 21/87	11	45.1 ± 6.0	2.75 ± 1.28	63.6	18.2	36.4	9.1	90.0	10.0
Booker Lagoon	Oct. 6/87	5	50.0 ± 2.5	4.00 ± 1.10	80.0	60.0	40.0	40.0	80.0	0.8
Tzartus Island	June 23/87	36	46.2 ± 3.5	4.00 ± 0.97	88.9	61.1	38.9	61.1	12.9	70.8
Tzartus Island	Aug. 26/87	21	49.8 ± 4.9	3.51 ± 1.16	52.4	19.4	42.9	14.3	25.0	39.0
Tzartus Island	Oct. 6/87	16	51.5 ± 3.2	4.52 ± 1.07	93.8	6.3	18.8	0.0	6.3	26.7
Tzartus Island	Feb. 9/87	18	54.9 ± 7.1	4.85 ± 2.28	44.4	22.2	44.4	27.8	11.1	-
Trevenen Bay	June 25/87	46	46.2 ± 5.9	3.36 ± 1.15	78.3	50.0	47.8	65.2	0.0	86.7
Trevenen Bay	Aug. 19/87	28	48.7 ± 6.9	3.19 ± 1.39	57.1	57.1	32.1	32.1	34.8	52.3
Trevenen Bay	Oct. 9/87	17	51.7 ± 4.0	2.72 ± 1.10	11.8	11.8	17.6	5.9	0.0	24.2
Indian Arm	Aug. 19/87	50	50.5 ± 4.7	4.42 ± 4.90	54.0	40.0	56.0	14.0	5.3	84.4
Indian Arm	Oct. 9/87	29	46.8 ± 5.9	3.68 ± 1.27	72.4	24.1	27.6	0.0	17.9	76.6
Indian Arm	Feb. 12/88	50	53.9 ± 4.6	5.59 ± 1.73	84.0	34.0	56.0	62.0	6.0	-

[1] Presented as mean ± standard deviation.

[2] Identified microscopically (100-400X magnification) as mussels with numerous fully developed gametes in mantle tissue.

[3] Mussels with ≥40% of the cells with neoplastic characteristics on stained preparation of haemocytes in body fluids were considered as having advanced neoplasia.

[4] Data from G. Jamieson (pers. comm.).

Table 5. Mortality and condition of survival mussels (Mytilus edulis) held in experimental tanks at various temperatures and salinities for 107 days (9 December to 26 March).

Group	Temp. °C	Sal. °/oo	Total mussels (3 tanks)	Accumulative mortality	Condition of survivors[1]								
					Sample size	Shell length (cm)	Meat wet weight (g)	Tissue categories[2]		Reproductive status			
								1	3	male	female	unknown	gravid
A	9.0	31	151	19.2	110	4.1±0.39	1.9±0.81	44.5	13.6	27.3	40.0	32.7	15.5
B	19.5	22	156	25.6	93	4.2±0.48	1.9±0.66	48.4	15.1	35.5	39.8	24.7	4.3
C	9.0	22	149	18.1	116	4.1±0.39	2.5±0.88	58.6	6.0	31.9	50.0	18.1	20.7
D	19.5	31	155	63.9	31	4.1±0.39	1.1±0.45	32.2	6.5	25.8	35.5	38.7	0

[1]Exclusive of mussels examined histologically. Shell length and meat wet weight are presented as mean ± standard deviation and tissue categories and reproductive status are presented as percentages.

[2]Tissues were categorized as: 1- tissues filling shell with good colour and 3- tissues thin and watery (category 2 represents an intermediate state for which results are not listed herein).

Table 6. Mortality and prevalence of haemocytic neoplasia in mussels[1] (Mytilus edulis) held in experimental tanks at various temperatures and salinities.

Group	Temp.[2] °C	Salinity[2] °/oo	Incubation period (days)	Percent dead[3]	Mussels with neoplastic haemocyte intensity[4] light	heavy	Additional mussels with no neoplasia but tissue infiltration by normal haemocytes
			1 to 21	6.6	4	2	6
A	9.0	31	22 to 50	3.3	0	3	7
	(8.0-9.5)	(none)	51 to 80	6.6	2	1	5
			81 to 107	2.6	2	3	3
			1 to 21	12.8	3	1	4
B	19.5	22	22 to 50	0.6	0	3	2
	(17.5-22.0)	(20-24)	51 to 80	4.5	1	0	0
			81 to 107	7.7	0	1	1
			1 to 21	5.4	0	2	4
C	8.8	22	22 to 50	3.4	0	1	7
	(8.0-9.0)	(none)	51 to 80	4.7	3	2	1
			81 to 107	4.7	1	2	3
			1 to 21	20.0	0	2	7
D	20.0	31	22 to 50	16.8	0	0	1
	(18.0-22.0)	(30-31)	51 to 80	14.2	1	0	0
			81 to 107	12.9	0	0	2

[1]Twenty-five mussels were examined for each group of tanks on the last day of each incubation period except for group B and C on day 21 when 23 mussels were examined for each group and group D on day 80 when 14 mussels were examined.

[2]Expressed as mean followed by range of variation in parenthesis.

[3]Calculated at end of incubation period from mussels in 3 unsampled tanks for each group (see Table 5 for initial number of mussels in these tanks for each group).

[4]Intensity of haemocytic neoplasia was determined by examining stained haemocyte preparations and histological sections of each mussel. Light intensities occurred in mussels with 10-40% of the cells on haemocyte preparation neoplastic and/or few neoplastic cells observed histologically. Heavy intensity occurred in mussels with ≥40% of the cells on haemocyte preparation neoplastic and/or in numerous neoplastic cells were observed in histological tissue sections.

Table 7. Comparison between haemocyte preparations and histological examinations for the detection of neoplasia[1] in 385 mussels (Mytilus edulis).

Method of examination	Number of mussels with neoplasia			Number of preparations unreadable
	heavy	light	negative	
Haemocyte prep.	24	8	332	21
Histological	23	17	345	0
By haemocyte prep. but not histology	2	0	-	-
By histology but not haemoctye prep.	1	9	-	-
By both techniques	22	8	-	-

[1]Intensity of neoplasia in haemocyte preparations was considered heavy if ≥40% of the cells were neoplastic and light if 10 to 40% of the cells were neoplastic.

Intensity of neoplasia in histological preparations was considered heavy if there was an abundance of haemocytes, of which the majority were neoplastic, in most tissues and light if haemocytes in some tissues were more abundant than normal and if some of these cells were neoplastic.

Table 8. Mortality and prevalence of haemocytic neoplasia in mussels (Mytilus edulis) held in experimental tanks for 175 days at cool (winter) or warm ambient (summer) temperatures.

Water condition[1]	Total mussels	Percent dead	Size of survivors[2]		Prevalence (%) of neoplastic haemocytes		
			Shell length (cm)	meat wet weight (g)	Total examined	Intensity[3]	
						light	heavy
Warm	202	88.6	4.2+0.4	1.7+0.5	23	26.1	17.4
Cool	200	73.5	4.2+0.4	1.9+0.7	53	9.4	20.8
Warm	170	71.8	1.3+0.2	-[4]	20	20.0	35.0
Cool	178	61.2	1.4+0.3	-	20	35.0	50.0

[1]See Fig. 8 and 9 - for records of temperatures and salinities.

[2]Mean ± standard deviation, measured at termination of experiment.

[3]Intensity of haemocyte neoplasia was determined by examining histological sections of 20 mussels from each group and by examining haemocyte preparations of the additional large mussels. Light intensities occurred in mussels if haemocytes in some tissues were more abundant than normal and if some of these cells were neoplastic or if 10 to 40% of the cells on haemocyte preparations were neoplastic. Heavy intensities occurred in mussels with more than the above mentioned numbers of neoplastic haemocyte in tissue sections or in haemocyte preparations.

[4]-=not measured.

Table 9. Mortality and prevalence of haemocytic neoplasia in surviving mussels (Mytilus edulis) 185 days following inoculation with 1.9×10^5 neoplastic haemocytes (challenged) or autoclaved seawater (control).

			Size of survivors[1]		Mussels with neoplastic haemocytes		
						Intensity[2]	
Group	Total mussels	Percent dead	Shell length (cm)	Meat wet weight (g)	Total examined	light	heavy
Challenged	46	73.9	4.7+0.3	2.4+0.7	12	3	6
Control	53	75.5	4.7+0.3	2.1+0.8	13	1	1

[1]Mean ± standard deviation, measured at termination of experiment.

[2]Intensity of haemocyte neoplasia was determined by examining histological sections. Light intensities occurred in mussels if haemocytes in some tissues were more abundant than normal and if some of these haemocytes were neoplastic. Heavy intensities occurred in mussels with numerous neoplastic haemocytes.

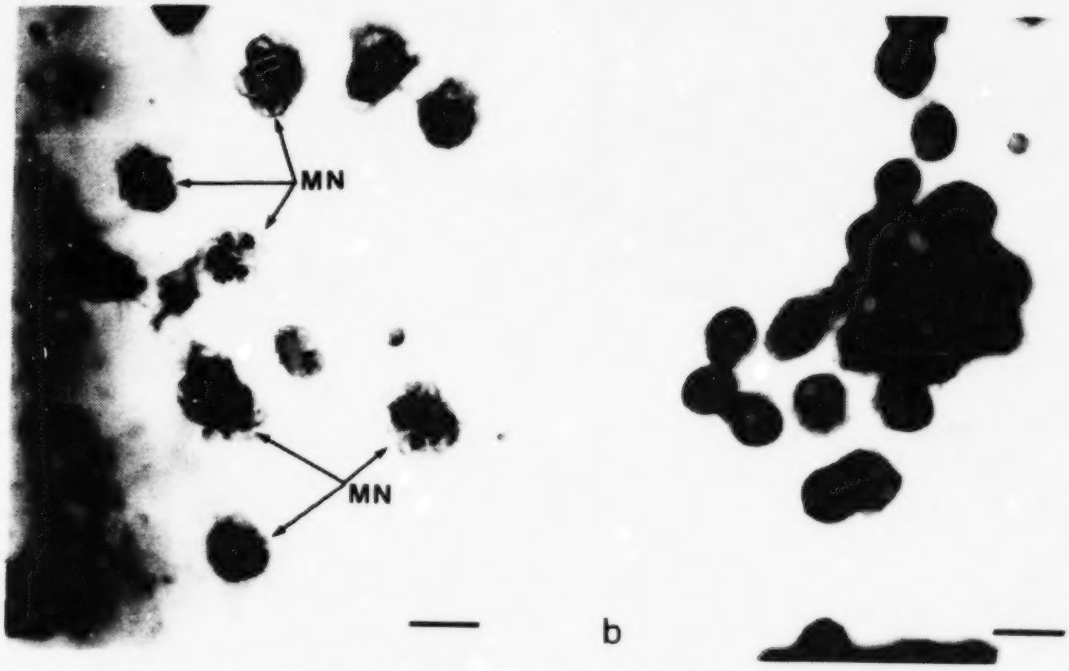

Fig. 1. Neoplastic haemocytes of Mytilus edulis in haemocyte preparations stain illustrating several roundish cells each with a multilobed nucleus (MN) from one mussel (a) and more classical large unlobed nucleate cells from another mussel (b) (Schiff (Feulgen) stain, scale bar = 10 μm).

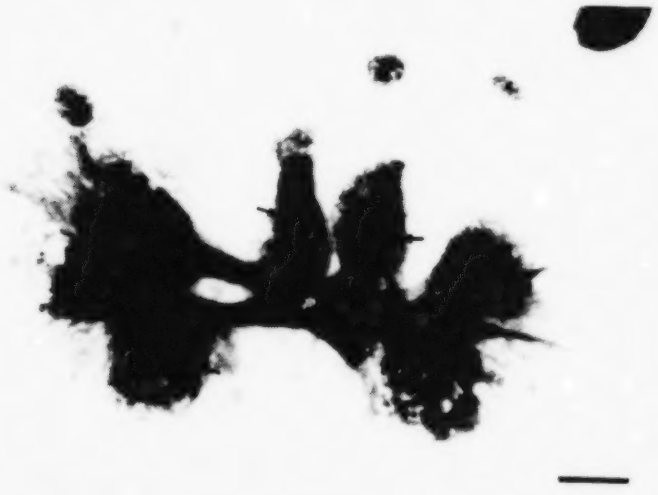

Fig. 2. Normal haemocytes with spreading cytoplasm from Mytilus edulis in a haemocyte preparation. Note the small size of the nucleus (arrows) in comparison to the amount of cytoplasm (Schiff (Feulgen) stain, scale bar = 10 μm).

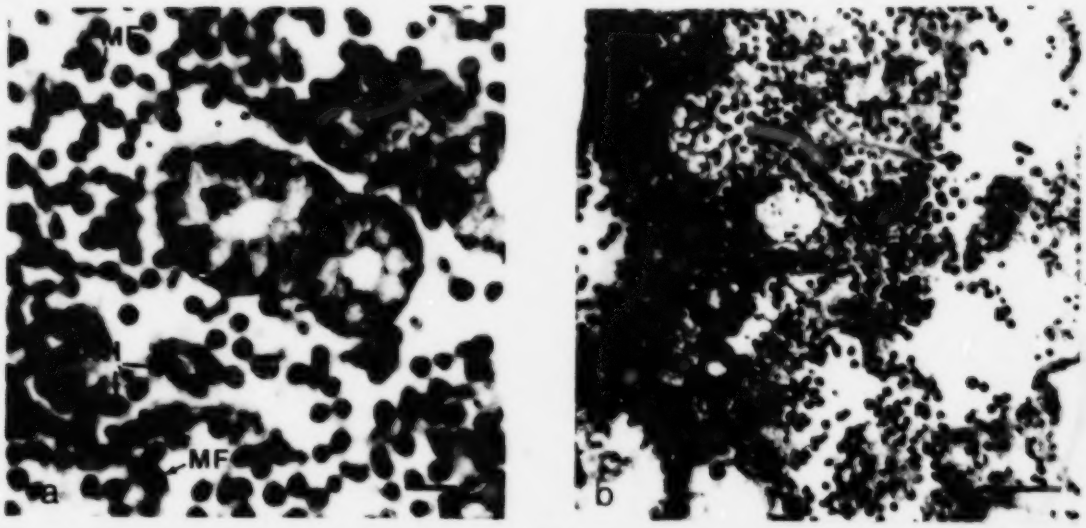

Fig. 3. Histological sections through the digestive gland (a) and mantle (b) of Mytilus edulis with a heavy intensity of haemocytic neoplasia (haematoxylin and eosin stain). In figure a, note the abundance of abnormal haemocytes (AH), each with a large nucleus containing two or more nucleoli and a small amount of peripheral cytoplasm. Also, a few haemocytes are undergoing mitosis (MF, scale bar = 25 μm). In figure b, some displacement of normal tissues has occurred and there is no evidence of gamete development (scale bar = 50 μm).

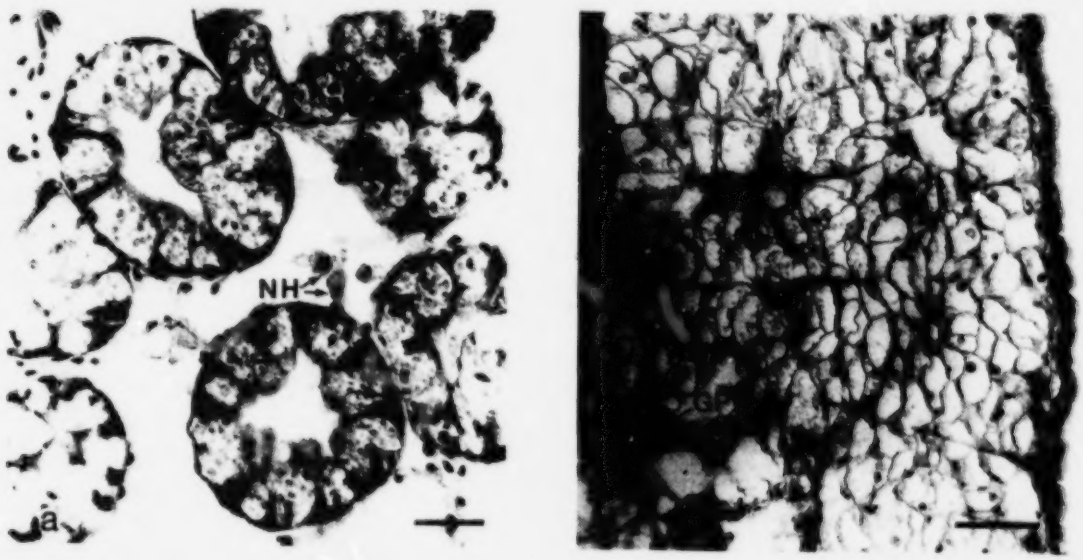

Fig. 4. Histological sections through the digestive gland (a) and mantle (b) of normal Mytilus edulis (haematoxylin and eosin stain). In figure a, note the presence of only a few normal haemocytes (NH) in the spaces between the tubules (scale bar = 25 μm). In figure b, the gamete primordia (GP) is at a very early stage of development (scale bar = 50 μm).

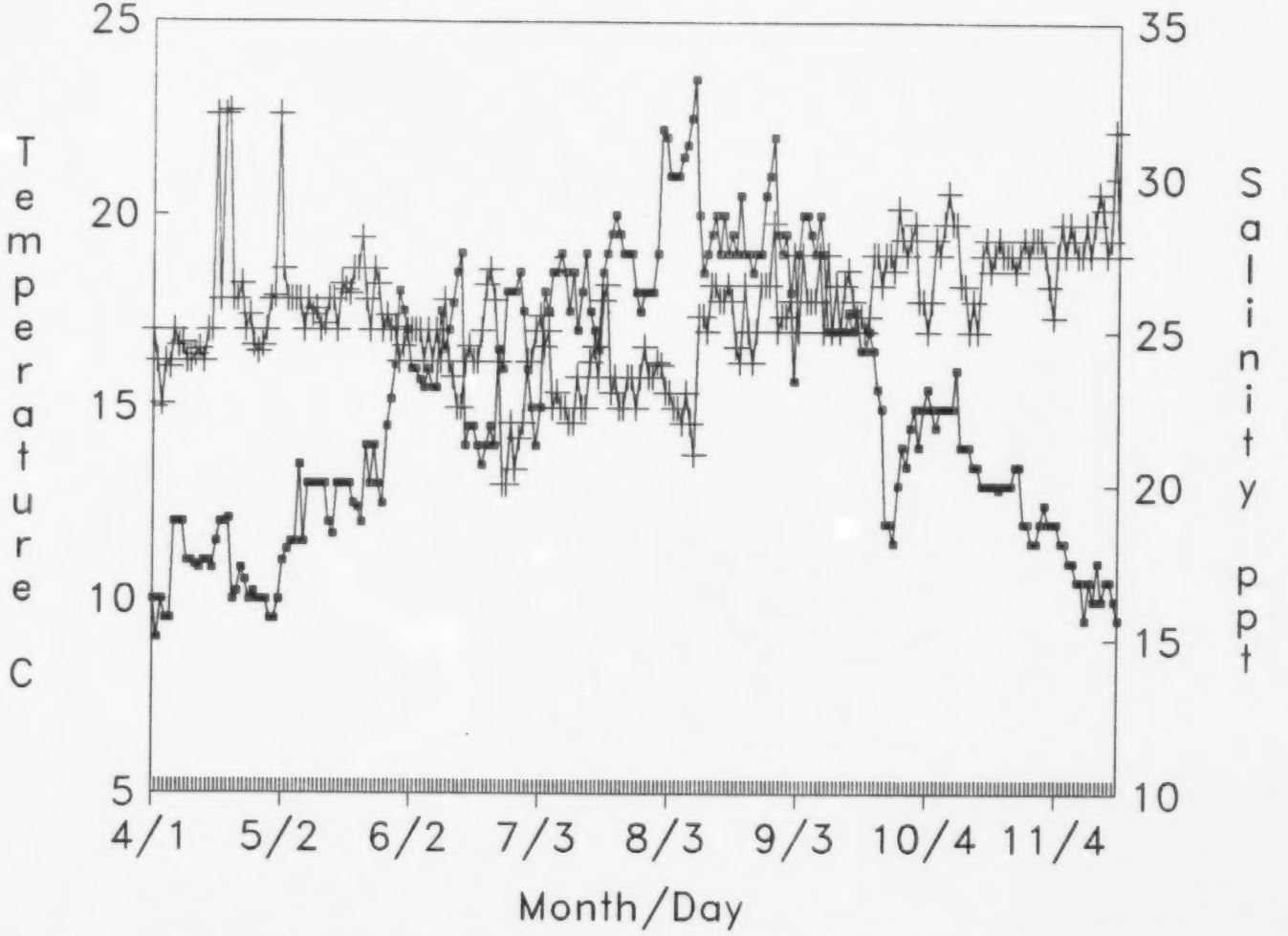

Fig. 5. Temperature (squares) and salinity (crosses) profiles of surface water at the Brandon Island experimental netpen fish culture facility in Departure Bay from April to November 1986.

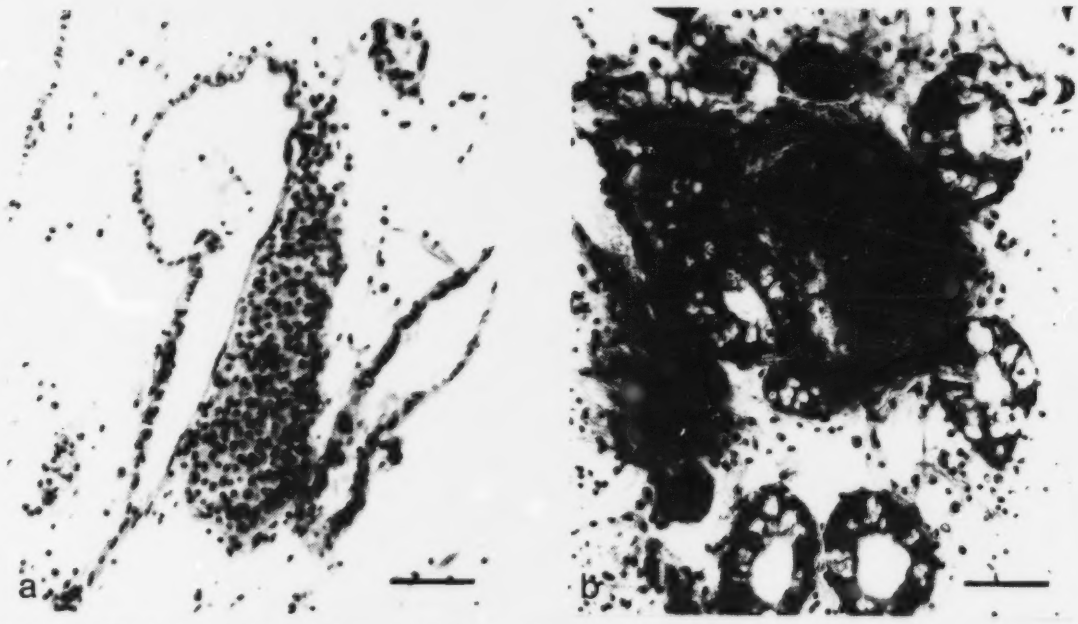

Fig. 6. Histological sections through an unusual accumulation of normal looking haemocytes in a patch (a) and in a tight whorl-like pattern (b) in the connective tissue of Mytilus edulis with no evidence of haemocytic neoplasia (haematoxylin and eosin stain, scale bars = 50 μm).

Fig. 7. Histological sections through the intestine of Mytilus edulis with an unusually large clump (a, scale bar = 100 μm) and a higher magnification of two smaller whorls (b, scale bar = 25 μm) of normal haemocytes between the epithelial cells apparently in the process of diapedesis (haematoxylin and eosin stain).

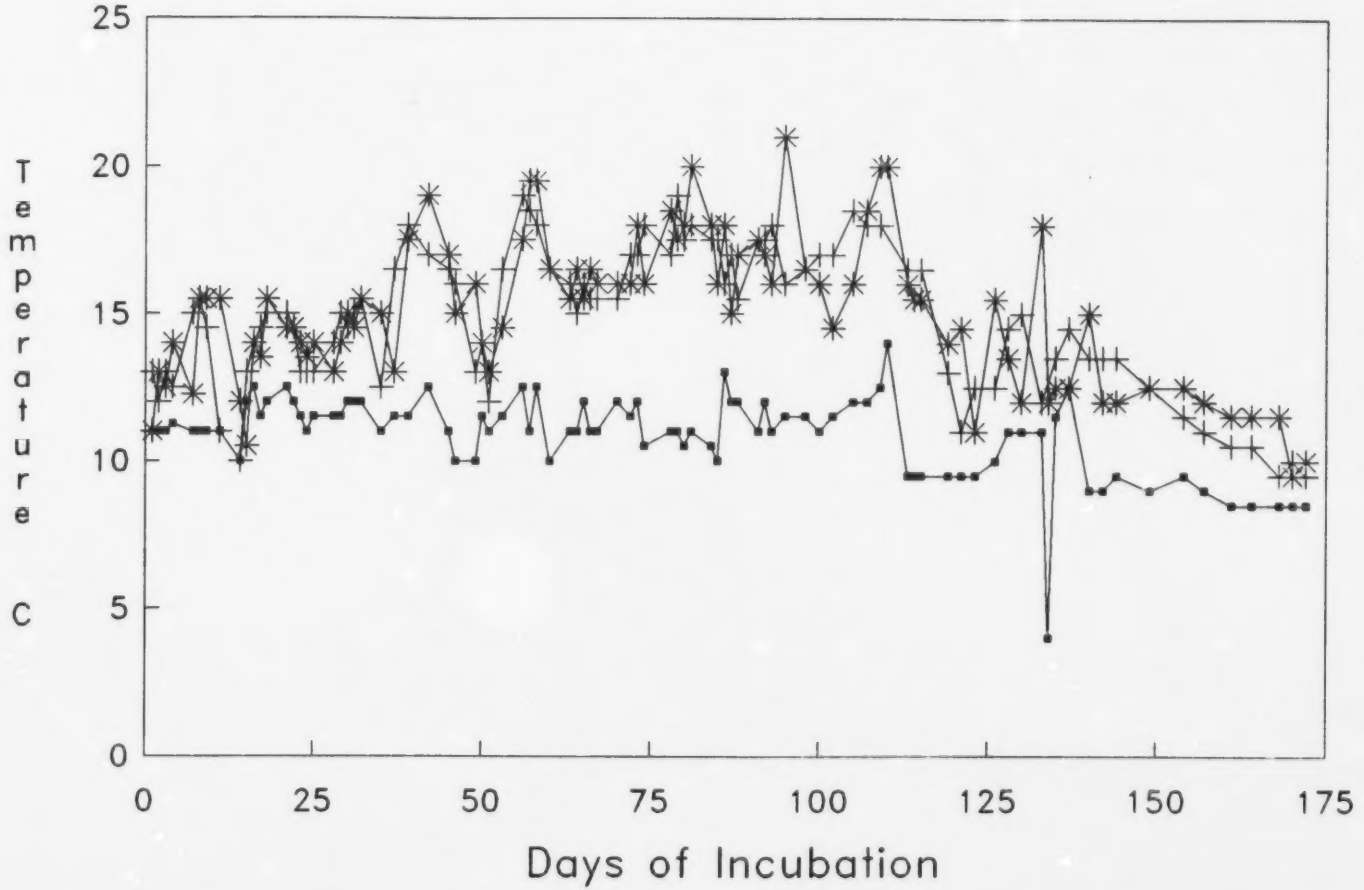

Fig. 8. Record of water temperature in tanks maintained at cool temperatures (squares) and tanks adjusted to approximate temperatures (asterisks) measured off the wharf in Departure Bay (crosses) during Investigation 5 from 18 May to 9 November 1987.

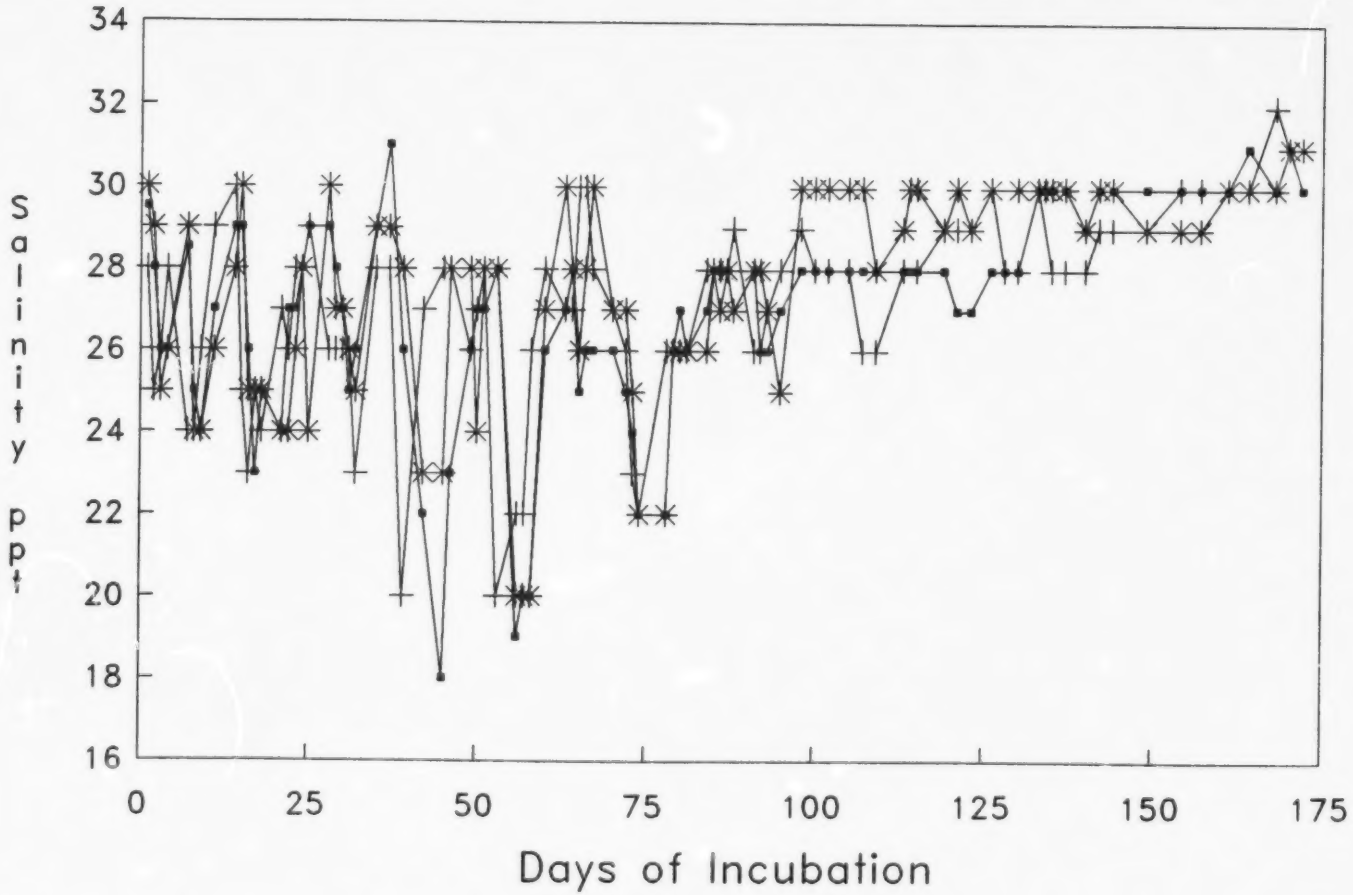

Fig. 9. Record of water salinity in tanks maintained at cool temperatures (squares) and tanks adjusted to approximate salinities (asterisks) measured off the wharf in Departure Bay (crosses) during Investigation 5 from 18 May to 9 November 1987.

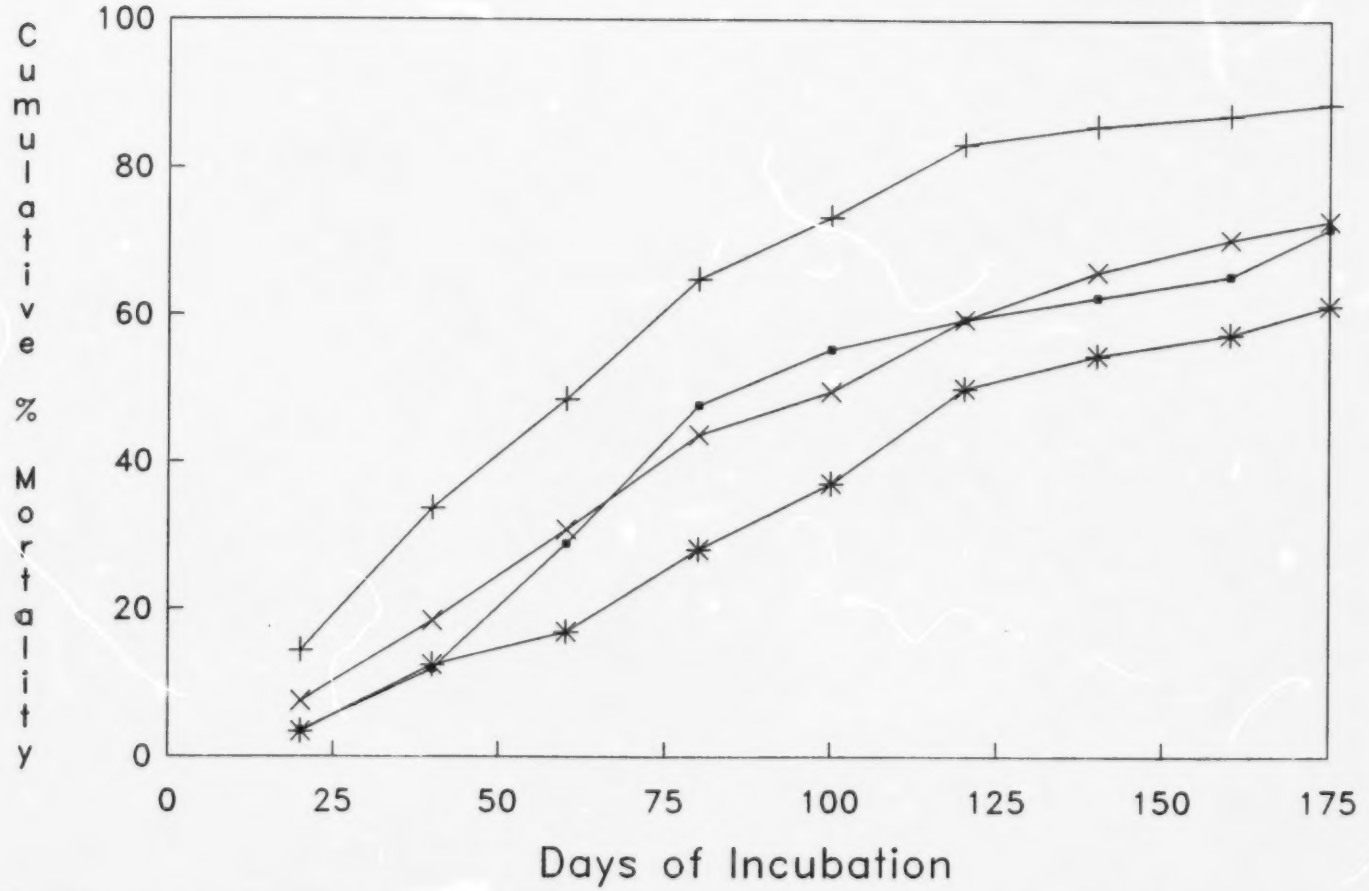

Fig. 10. Cumulative mortalities of large mussels held at ambient temperatures (crosses), large mussels held at cool temperatures (Xs), small mussels held at ambient temperatures (squares), and small mussels held at cool temperatures (asterisks) during Investigation 5.

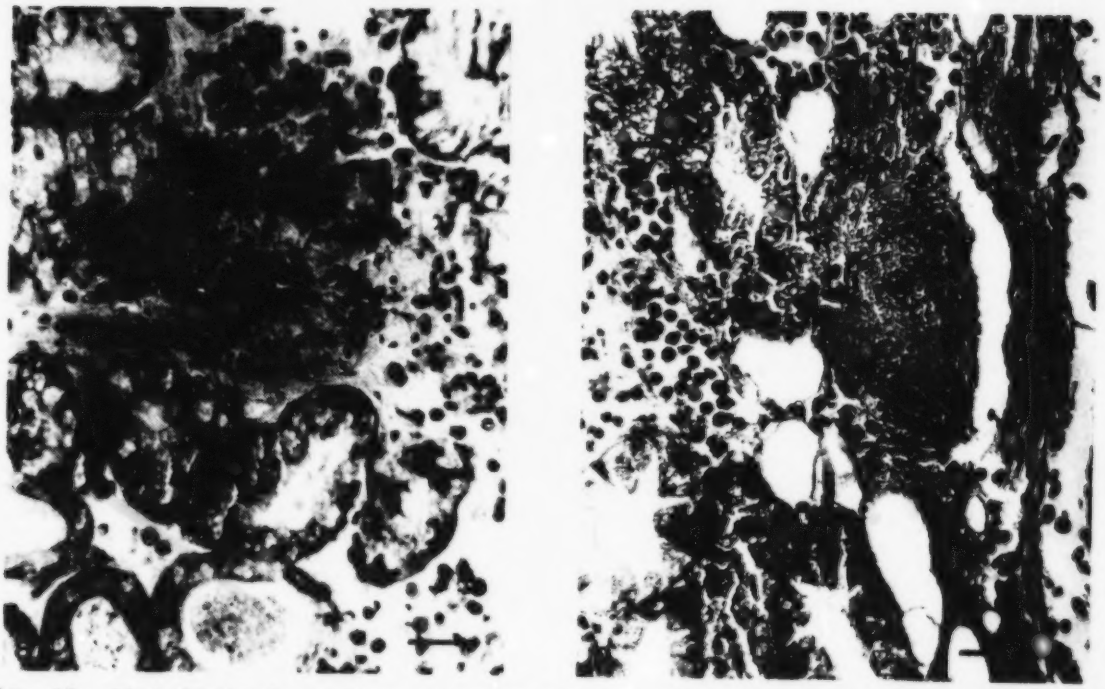

Fig. 11. Histological sections through the digestive gland area of Mytilus edulis with haemocytic neoplasia (haematoxylin and eosin stain). Figure a illustrates a whorl of normal and neoplastic haemocytes found in the connective tissue of one mussel (scale bar = 25 μm) and figure b illustrates a whorl of normal haemocytes that does not incorporate the surrounding neoplastic haemocytes (scale bar = 50 μm).

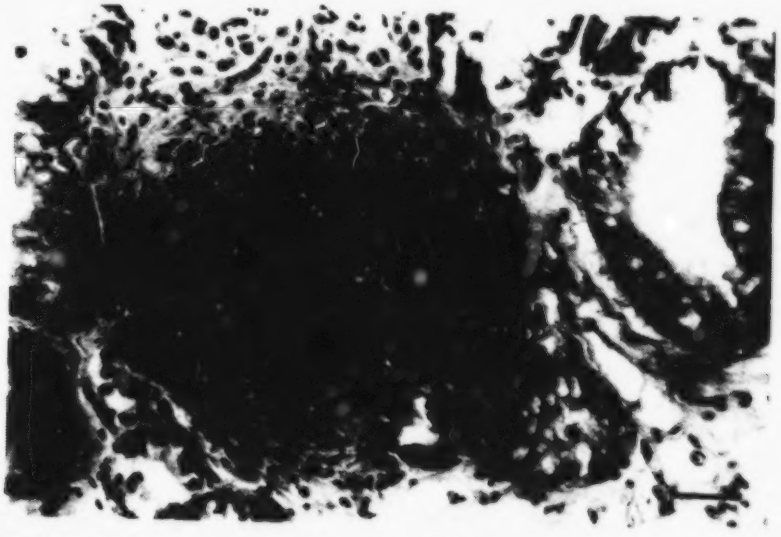

Fig. 12. Histological section of the digestive gland area of Mytilus edulis through a cyst or whorl of normal haemocytes containing necrotic cells and cells with eosinophilic inclusions of unknown aetiology (haematoxylin and eosin stain, scale bar = 25 μm).

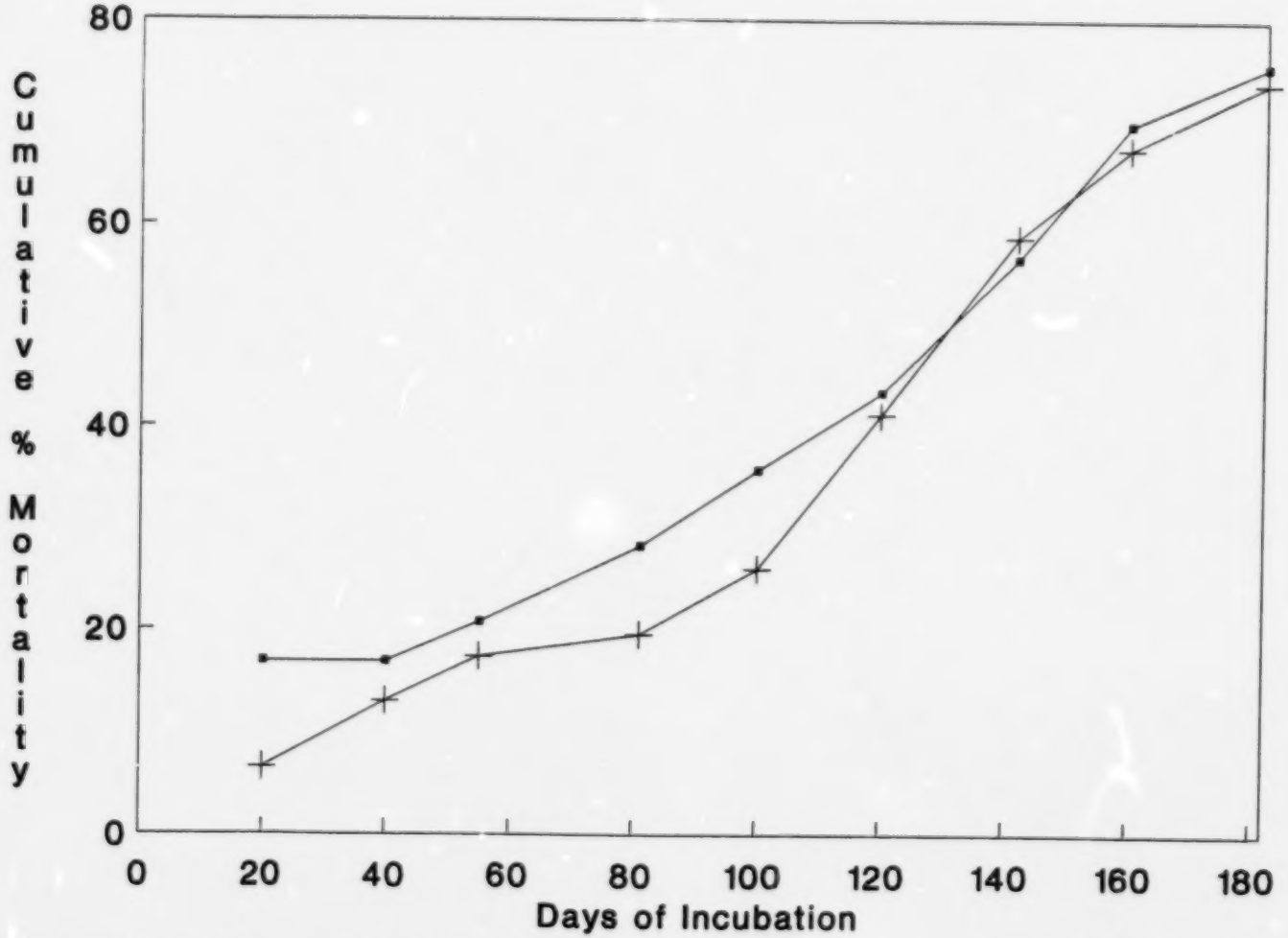

Fig. 13. Cumulative mortalities of Mytilus edulis inoculated with about 1.9 x 10^5 neoplastic haemocytes (crosses) and control mussels (squares) held in ambient seawater from 16 February to 16 August 1987 during Investigation 6.

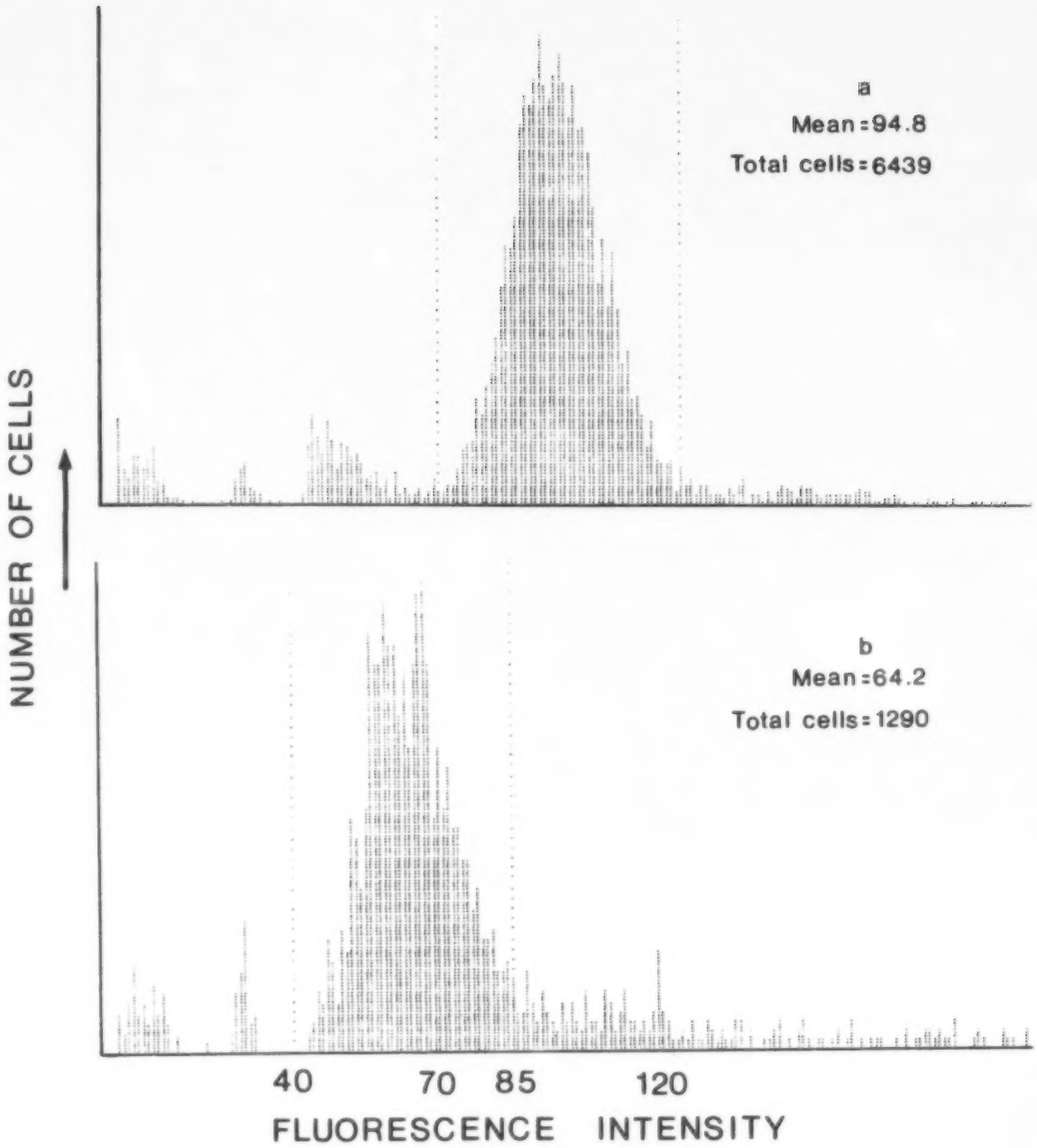

Fig. 14. Fluorescence intensity for haemocytes from a Mytilus edulis with a heavy intensity of haemocytic neoplasia (a, above) and a normal mussel (b, below) using propidium iodide stain and flow cytometery. The bars on either side of the main peaks are channel numbers which reflect increasing fluorescent intensity. The mean fluorescence and total cells counted is presented on each plot. The first few small peaks at the beginning of each plot illustrates nonspecific fluorescence of debris or broken haemocytes damaged during preparation.

APPENDIX I

METHOD OF REPEAT SAMPLING OF HAEMOCYTES IN *Mytilus edulis*

The following procedure was modified from that of Farley et al. (1986). During the sampling process, mussels were held on damp paper towels placed on frozen ice packs. The area of the shell over the posterior adductor muscle of one valve was swabbed with 100% isopropanol. With a sterile scalpel blade, a small puncture (approximately 1 mm in diameter) was scraped in the shell anterior to the adductor muscle, over the heart. Using a 3 ml syringe equipped with a 20 gauge, 1 inch (2.5 cm) needle, about 0.1 ml of haemolymph was drawn into 2.4 ml of cold sterile artificial sea water. (The haemolymph was diluted in sterile sea water in an attempt to reduce clumping of the haemocytes.) The haemolymph and sea water was immediately injected through the parafilm cover into a chamber attached to a Poly-L-lysine coated microscope slide (Appendix III) cooled to about 5°C. The preparation was incubated at about 5°C for 1 hr. After incubation, the sea water and chamber were removed from the slide and without drying, the slide with attached cells was immersed in 100% methanol for three min then transferred to 10% buffered formalin (Lehmann 1964) for three min or for storage for up to eight days. The slide was stained and processed for permanent storage as described in Appendix III.

After withdrawing the sample of haemolymph, a drop of melted paraffin was placed on the shell to seal the puncture. When the paraffin set, the mussel was returned to sea water.

REFERENCES

Farley, C. A., S. V. Otto, and C. L. Reinisch. 1986. New occurrences of epizootic sarcoma in Chesapeake Bay soft shell clams *Mya arenaria*. Fish. Bull. U.S. 84: 851-857.

Lehmann, D. L. 1964. A method for the critical staining of culture trypanosomes. Trans. R. Soc. Trop. Med. Hyg. 58: 366.

APPENDIX II

METHOD OF SAMPLING HAEMOCYTES OF TERMINALLY SAMPLED *Mytilus edulis*

The mussel was carefully shucked by removing one valve while maintaining the mantle from that valve with the body that was still attached to the other valve. A micro haemotocrit capillary tube (length 75 mm, ID 1.2 mm) was used to withdraw about 0.05 ml of haemolymph from the heart area located just anterior to the posterior adductor muscle. The haemolymph was immediately blown from the tube into a drop (about 0.05 ml) of sterile sea water on a poly-L-lysine coated microscope slide (Appendix III). Three separate samples from three mussels could be placed in a row on each frosted end microscope slide with appropriate label. The slide was incubated in a humidity chamber at about 5°C for 10 min followed by immersion in 10% buffered formalin (Lehmann 1964) for three min or for storage for up to eight days. The slide was stained and processed for permanent storage as described in Appendix III.

REFERENCES

Lehmann, D. L. 1964. A method for the critical staining of culture trypanosomes. Trans. R. Soc. Trop. Med. Hyg. 58: 366.

APPENDIX III

PREPARATION OF MICROSCOPE SLIDES AND STAINING OF HAEMOLYMPH OF (*Mytilus edulis*) FOR DETERMINING INTENSITY OF HAEMOCYTIC NEOPLASIA

The procedure was modified from that of Farley (1969), Howard and Smith (1983), and Farley et al. (1986).

Prior to use, microscope slides were coated with Poly-L-lysine to improve the adhesiveness of neoplastic cells which are rounded *in vitro* and usually do not stick to glass. Prior to Poly-L-lysine coating, slides were cleaned by dipping them six to ten times in acid alcohol, followed by dipping in three changes of distilled water. Slides were then dipped in 0.1% Poly-L-lysine (MW 90,000) in distilled water and air dried. Dried slides were stored dust-free for up to 6 months prior to use.

Haemocytes from mussels that had been placed on Poly-L-lysine coated slides and fixed in 10% buffered formalin for three min to eight days (Appendix I and II) were stained and processed for permanent storage without being air dried as follows:

1) Distilled water - 2 changes - 1 min each
2) 5N HCl (prepared by mixing 292 ml of distilled water and 208 ml of concentrated Hcl sp.gr. 1.19) - 30 min
3) Distilled water - 2 changes - 1 min each
4) Refrigerated Schiff (Feulgen) stain (see Howard and Smith 1983 for preparation) - 15 min
5) Distilled water - 2 changes - 1 min each
6) Running tap water - 2 min
7) Distilled water - 1 min
8) 0.03% Picromethyl blue prepared by adding 0.15 gm Cotton blue C4B (Allied Chemicals) to 500 ml saturated aqueous acid (Fisher Scientific) - 3 min
9) 100% Isopropanol - 3 changes - 2 min each
10) Xylene - 2 changes - 2 min each
11) Mount coverslip with permount without allowing slide to air dry

REFERENCES

Farley, C. A. 1969. Probably neoplastic disease of the haematopoietic system in oysters, Crassostrea virginica and Crassostrea gigas. Nat. Cancer Inst. Monogr. 31: 541-555.

Farley, C. A., S. V. Otto, and C. L. Reinisch. 1986. New occurrence of epizootic sarcoma in Chesapeake Bay soft shell clams Mya arenaria. Fish. Bull. U.S. 84: 851-857.

Howard, D. W. and C. S. Smith. 1983. Histological techniques for marine bivalve mollusks. U.S. Dep. Commer. NOAA Tech. Memo NMFS F/NEC 25. 97 p.